

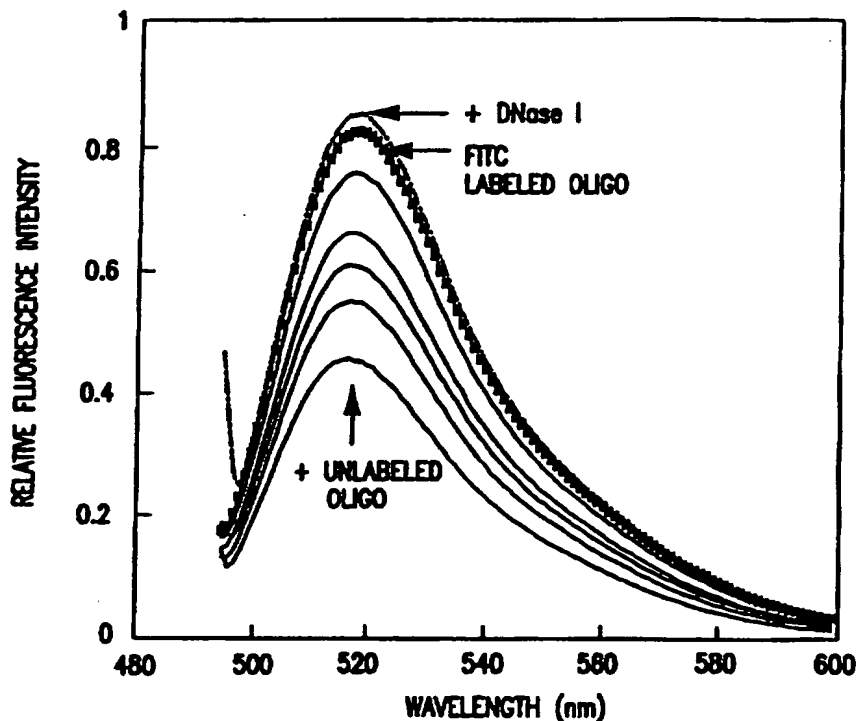


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(54) Title: FLUOROMETRIC ASSAY FOR DETECTING NUCLEIC ACID CLEAVAGE**(57) Abstract**

A method of detecting an enzyme-mediated DNA cleavage reaction in a fluorometric assay is provided. The method can be used to detect DNA cleavage caused by restriction endonucleases, retroviral integrase enzymes, DNases, RNases, or enzymes utilized in other strand separating processes in molecular biology.



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Fluorometric Assay for Detecting Nucleic Acid Cleavage

Background of the Invention

Field of the Invention

5 The present invention is in the fields of biochemistry and molecular biology. The invention relates to an assay for detecting nucleic acid cleavage reactions. More particularly, the invention relates to a continuous fluorometric assay for detecting nucleic acid cleavage reactions that are enzyme-mediated.

10 *Description of the Related Art*

 Virtually all protocols in molecular biology require, at some point, cleavage of nucleic acids into smaller sized discrete fragments. *In vitro* cleavage of nucleic acids is typically accomplished with restriction endonucleases. Restriction endonucleases are commercially available
15 enzymes, derived from bacteria, that recognize short DNA sequences and then cleave the double-stranded DNA at specific sites within, or adjacent to, the recognition sequence. These enzymes have been classified into three groups - Types I, II, and III. Type II restriction enzymes, which cleave a specific sequence of nucleotides and a separate methylase that modifies the same
20 recognition sequence, are widely used in molecular cloning. A partial list of restriction enzymes and their recognition sequences is provided in Chapter 5 of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, (1989).

 Restriction endonuclease cleavage of DNA into discrete fragments is
25 one of the most basic procedures in molecular biology. The cleavage sites provide specific landmarks for obtaining a physical map of DNA. Further, the ability to produce specific DNA fragments by cleavage with restriction enzymes makes it possible to purify these fragments by molecular cloning.

-2-

In addition, restriction enzymes have been utilized extensively for finding restriction fragment length polymorphisms (RFLPs) in allelic genomic regions. The use of RFLPs as genetic markers has been exploited in genetic linkage analysis, determination of patterns of inheritance for genetic disease, mapping
5 of genes to specific chromosomal loci, and genetic fingerprinting.

Many enzymes other than restriction endonucleases are routinely used in molecular cloning. For example, DNases, RNases, exonucleases, and helicases are utilized in molecular biology to effect strand separation or denaturation of nucleic acids. These enzymes are discussed generally in
10 Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, (1989). Such enzymes are utilized in numerous processes in molecular biology that serve to amplify and detect DNA, such as, polymerase chain reaction (PCR) (described in U.S. Patents
15 4,683,194, 4,683,195 and 4,683,202), ligase chain reaction (LCR) (described in published PCT application WO 89/09835), and catalytic hybridization amplification (CHA) (described in published PCT application WO 89/09284, and U.S. Patents 5,011,769 and 4,876,187).

Ascertaining that nucleic acid cleavage has occurred, and evaluating the efficiency of the cleavage process, have traditionally been done using a gel electrophoresis assay system (Sambrook *et al.*, *supra*). Such a system,
20 however, is not only time-consuming and laborious, but the assay is discontinuous, meaning that the process cannot be monitored throughout the cleavage process. This is clearly a disadvantage in certain situations, such as where partial cleavage is desired, or where one needs to establish precise
25 enzyme kinetic information. Further, the conventional assays are often inhibited by high concentrations of salt that may be required owing to the purification and solubility of the proteins involved. Finally, radioactive labeling of the substrates is often required to achieve the necessary level of sensitivity.

30 More recently, a continuous spectroscopic assay for endonucleases has been reported (Waters and Connolly, *Anal. Biochem* 204:204-209 (1992)).

This assay is based on the hyperchromic effect resulting from turnover of a duplex oligonucleotide substrate to single-stranded DNA products. Although this technique is continuous, its scope is limited by its narrow dynamic range and limited range of substrate concentrations.

5 A sensitive non-isotopic enzyme linked immunoabsorbent assay (ELISA) for determining the DNA cleavage activity of restriction endonucleases was described by Jeltsch *et al.*, *Anal. Biochem.* 213:234-240 (1993). This assay utilized DNA substrates that are labeled on both ends; one 5' end is labeled with biotin, and the other 5' end is labeled with fluorescein or digoxigenin. The use of biotin-labeled DNA in this assay renders the
10 method discontinuous and necessitates extensive sample handling for the detection step.

 Finally, endonuclease-catalyzed cleavage reactions of fluorophore-labeled oligonucleotides have been monitored by fluorescence resonance energy transfer (FRET) techniques (Ghosh *et al.*, *Nucleic Acids Res.* 22:3155-3159 (1994)).

 Fluorescence resonance energy transfer (FRET) (Forster, T., *Ann. Phys. (Leipzig)* 2:55-75 (1948); Stryer, L., *Annu. Rev. Biochem.* 47:819-846 (1978); Stryer, L., *Proc. Natl. Acad. Sci. USA* 58:719-726 (1967); Conrad and Brand, *Biochemistry* 7:777-787 (1968); Chen and Scott, *Anal. Lett.* 18:393 (1985); Wu and Brand, *Anal. Biochem.* 218:1-13 (1994)) is the
20 transfer of electronic excitation energy by the Förster mechanism, and measures the distance between a pair of fluorophores (donor and acceptor) in macromolecules, in the range of 10-80 Angstroms (Å). Cardullo *et al.*, *Proc. Natl. Acad. Sci. USA* 85:8790-8794 (1988), utilized FRET experiments to
25 study the hybridization of complementary oligodeoxynucleotides. Upon hybridization, energy transfer was detected by both a decrease in fluorescein (donor) emission intensity and an enhancement of rhodamine (acceptor) emission. Cooper and Hagerman, *Biochemistry* 29:9261-9268 (1990), also
30 utilized FRET to determine the interarm angles of a synthetic DNA four-way junction. However, these investigators reported that upon annealing of a

fluorescent-modified strand and its unlabeled complementary strand, the probe fluorescence was quenched (Clegg *et al.*, *Biochemistry* 31:4846-4856 (1992); Cooper and Hagerman, *supra*), and the wavelength of the emission spectrum was shifted upon the formation of duplex DNA. These results suggest that effects other than dipolar energy transfer mechanisms alter the donor fluorescence (in the presence *or* absence of acceptor at the ends of complementary strands), and that these effects must be examined in order to reliably measure distances in DNA molecules by FRET. Thus, the occurrence of nondipolar effects on fluorescently labeled DNA may distort the distances quantified by FRET in certain instances.

Thus, there exists a need in the art for a continuous, accurate, sensitive, and non-isotopic assay for detecting restriction enzyme mediated cleavage of nucleic acids.

Another class of enzymes that catalyze nucleic acid cleavage reactions are retroviral integrases. These enzymes are responsible for catalyzing the integration of viral DNA into the host organism's chromosomal DNA. Currently, the target of viral therapeutics is to screen compounds that inhibit these enzymes.

For example, one focus of AIDS research is to find specific inhibitors of each step in the replication cycle of the HIV retrovirus. Although progress has been made in targeting reverse transcription, parallel efforts in inhibiting other processes could lead to the development of new therapeutic agents. Retroviral integration is a particularly attractive target in the search for specific inhibitors due to the absence of any known cellular counterparts in the host. The combined use of antiviral drugs with different target specificities will facilitate the search for therapeutic intervention.

The currently established *in vitro* assay system for HIV DNA integration is based upon the detection of labeled ^{32}P integrated products either by electrophoresis or by biotin-avidin interaction (the substrate DNA being radiolabeled with ^{32}P at the 5' end and biotin at the 3' end). (Craigie *et al.*, *Nucleic Acids Res.* 19:2729-2734 (1991)). Unfortunately, these methods are

-5-

time consuming and often do not yield precise kinetic information. A sensitive and more rapid assay is desired in order to screen vast numbers of potential drugs and natural products. A rapid, sensitive, and continuous *in vitro* assay for screening the vast number of potential enzyme inhibitors is clearly needed to provide precise kinetic information necessary to determine the relative effectiveness of any inhibitor. Also, an assay is needed that would not be affected by high salt concentrations.

Thus, in light of the foregoing, there exists a need in the art for a continuous assay for accurately and sensitively detecting enzyme-mediated nucleic acid cleavage *in vitro*.

Summary of the Invention

The present invention overcomes disadvantages of the prior art by providing a method of detecting an enzyme-mediated nucleic acid cleavage reaction in a continuous fluorometric assay comprising the steps of:

a) preparing a fluorescently labeled oligonucleotide containing a nucleotide sequence recognizable by said enzyme, wherein said oligonucleotide acts as an enzyme substrate; (b) contacting said oligonucleotide of step a) with said enzyme in an amount sufficient to enzymatically cleave said oligonucleotide; and c) detecting a nucleic acid cleavage reaction by detecting an increase in fluorescence.

The invention also provides specific embodiments wherein the oligonucleotide is fluorescently labeled at one or both ends.

The invention also provides specific embodiments wherein the method of detecting an enzyme-mediated nucleic acid cleavage reaction is employed in a catalytic hybridization amplification procedure, or a polymerase or ligase chain reaction.

The invention also provides preferred embodiments wherein the nucleic acid cleavage reaction is mediated by a restriction enzyme, DNase, RNase, or retroviral integrase enzyme.

-6-

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed.

For brevity, the following abbreviations are used throughout this application. FITC, fluorescein-5-isothiocyanate; EITC, eosin isothiocyanate; *Bam*HI, *Bacillus amyloliquefaciens* H; DNase I, Deoxyribonuclease I; HIV-1, human immunodeficiency virus type 1; IN, integrase (integration protein); EDTA, ethylenediaminetetraacetic acid; FRET, fluorescence resonance energy transfer; HPLC, high-performance liquid chromatography; DMF, dimethylformamide; 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; DAS, decay associated spectra; PAGE, polyacrylamide gel electrophoresis.

Brief Description of the Figures

Figure 1 is a graph depicting the fluorescence emission spectra of an FITC-labeled oligonucleotide. Changes in fluorescence intensity were recorded due to varying degrees of annealing of a 14-mer FITC-labeled oligonucleotide with its unlabeled complementary strands. Fixed concentrations (0.137 pmol) of the FITC-labeled oligonucleotide were annealed with 0.03 pmol, 0.06 pmol, 0.09 pmol, 0.117 pmol, and 0.15 pmol of the unlabeled complementary strand in 0.5 mL reaction volumes and their fluorescence intensities compared. Excitation wavelength was 490 nm.

Figures 2A and 2B: Figure 2A is a graph depicting the DAS of the FITC-labeled single-strand oligonucleotide (0.27 μ M).

Figure 2B is a graph depicting the DAS of the FITC-labeled oligonucleotide (0.27 μ M) annealed with its unlabeled complementary strand (0.4 μ M). Both experiments were performed in 50 mM Tris, pH 8.0, 10 mM MgCl₂, and 0.1 M NaCl at 25°C.

Figures 3A and 3B: Figure 3A is a graph depicting the emission spectra of FITC-labeled DNA substrates in the presence and absence of *Bam*HI endonuclease at 25°C.

-7-

Figure 3B is a graph depicting the emission spectra of FITC-labeled DNA substrates in the presence and absence of *Bam*HI endonuclease at 37°C. Identical concentrations of annealed oligonucleotides (FITC-labeled and unlabeled complementary strand) were cleaved with the *Bam*HI restriction endonuclease in 50 mM Tris, pH 8.0, 10 mM MgCl₂, and 0.1 M NaCl and the emission spectra were recorded with an excitation wavelength at 490 nm. The spectra of the initial annealed oligonucleotides (top curve) and the enhanced fluorescence following cleavage (bottom curve) are shown.

Figure 4 is a graph depicting the decay-associated spectra of FITC-labeled DNA substrate after the cleavage reaction by the *Bam*HI restriction endonuclease. The experiment was performed in 50 mM Tris, pH 8.0, 10 mM MgCl₂, and 0.1 M NaCl at 25°C.

Figure 5 is a graph depicting the temperature-dependent fluorescence intensity ratio of double-strand and single-strand FITC-labeled oligonucleotides. The thermally induced fluorescence changes were monitored at 520 nm with an excitation wavelength at 490 nm.

Figures 6A and 6B: Figure 6A is a graph depicting the enzyme concentration-dependent *Bam*HI cleavage of FITC-labeled DNA substrates. Curves A through D represent the kinetics of DNA cleavage by 10, 20, 40, and 60 units of *Bam*HI, respectively. The kinetic experiments were performed with 0.5 μM DNA substrate in 420 μl of 50 mM Tris, pH 8.0, 10 mM MgCl₂, and 0.1 M NaCl at 37°C. Fluorescence intensity was monitored with excitation and emission wavelengths of 520 nm and 490 nm, respectively.

Figure 6B depicts the initial velocities of cleavage reactions determined from the linear portions of the kinetic data and plotted as a function of enzyme concentrations.

Figures 7A and 7B: Figure 7A is a graph depicting a *Bam*HI cleavage reaction as a function of DNA substrate concentration. The graph shows a fluorometric analysis of the kinetics of a *Bam*HI cleavage reaction (10 units in 100 μl of 50 mM Tris, pH 8.0, 10 mM MgCl₂, and 0.1 M NaCl at 37°C). Curves A through E represent DNA cleavage reactions with 0.21, 0.36, 0.72, 1.08, and 0.18 μM DNA substrates, respectively. Curve E

depicts the cleavage reaction in the presence of 25 mM EDTA. Each reaction was stopped after 11 minutes by the addition of 25 mM EDTA.

5 **Figure 7B** is a photograph of the same reaction mixtures described in **Figure 7A**, run on 20% PAGE. The photograph was taken under ultraviolet illumination using a Kodak green filter without ethidium bromide staining.

Figure 8 is a graph depicting steady-state emission spectra of FITC and EITC labeled oligonucleotides. The spectra of F-D1/T1 (Curve A) and E-D2 (Curve B) were recorded with an excitation wavelength at 460 nm.

10 **Figures 9A and 9B:** **Figure 9A** is a graph depicting steady-state emission spectra of a fluorogenic substrate in the presence and absence of DNase I, in the presence of Mg^{2+} . Curve a depicts the emission spectrum of 4 pmol of a fluorogenic substrate in 400 μ l reaction buffer containing 25 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM DTT, and 5% glycerol at 37°C. Curve b depicts the emission spectrum of the same substrate digested with
15 DNase I. Curve c depicts the difference emission spectrum of curve b and curve a. Emission spectra were recorded with an excitation wavelength at 460 nm.

20 **Figure 9B** is a graph depicting steady-state emission spectra of a fluorogenic substrate in the presence and absence of DNase I, in the presence of Mn^{2+} . Curves a-c are the same as described for **Figure 9A**. Emission spectra were recorded with an excitation wavelength at 4460 nm. Inset: Peak normalized emission spectra of DNase I digested fluorogenic substrate in the presence of Mg^{2+} and Mn^{2+} .

25 **Figures 10A and 10B:** **Figure 10A** is a graph depicting steady-state emission spectra of a fluorogenic substrate in the presence and absence of HIV-IN. Curve A depicts the emission spectrum of 4 pmol of fluorogenic substrate in 400 μ l reaction buffer containing 25 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM DTT, and 5% glycerol at 37°C. Curve B depicts the emission spectrum of the same substrate digested with 40 pmol HIV-IN for 1 hour.
30 Curve C depicts the difference emission spectrum of curve B and curve A. Emission spectra were recorded with an excitation wavelength at 460 nm.

Figure 10B depicts the peak normalized emission spectra of F-D1/T1 and the difference spectrum (curve C).

Figures 11A, 11B, and 11C: Figure 11A is a graph depicting the kinetics of an HIV-IN cleavage reaction of a fluorogenic substrate monitored by FRET. The DNA cleavage reaction was initiated by the addition of HIV-IN to a preincubated reaction mixture containing 4 pmol of substrate. The conditions for the reactions are the same as in Figure 10. Changes in fluorescence intensity were monitored with excitation and emission wavelengths of 460 and 510 nm, respectively. Mn^{2+} quenched the fluorescence intensity of the substrate in the absence of the enzyme; thus, the kinetic data presented in this figure were intensity normalized according to the intensity ratio of the substrate in the presence of Mg^{2+} and in the presence of Mn^{2+} .

Figure 11B depicts the kinetics of an HIV-IN cleavage reaction of a fluorogenic substrate monitored by denaturing polyacrylamide gel electrophoresis (PAGE). The time course of a ^{32}P -5'-labeled fluorescent substrate was determined by radiographic assays. Two reactions in parallel with 0.15 pmol substrate were reacted with 4 pmol HIV-IN in 15 μ l reaction buffer at 37°C. The reactions were stopped at 1', 2.5', 5', 10', 15', 30' and 60' by addition of equal volume of stop solution. Reaction mixtures were analyzed by denaturing gel electrophoresis. Results were quantitated by utilizing a Hewlett-Packard ScanJet IIP and the densitometry program Scan Analysis 68020 (BioSoft). The line indicates the mean of the two experiments bounded by the actual values.

Figure 11C depicts the kinetics of the DNase I cleavage reaction.

Detailed Description of the Preferred Embodiments

The present invention is directed to a method of detecting an enzyme-mediated nucleic acid cleavage reaction using a continuous fluorometric assay. Generally, the present method employs a fluorescently-labeled oligonucleotide substrate containing a nucleotide sequence that is recognizable by the enzyme

-10-

that will catalyze the cleavage reaction. For example, in the case of a restriction enzyme, the oligonucleotide will contain the restriction site recognized by that restriction enzyme.

5 The oligonucleotide substrate can be DNA or RNA, and may be single or double-stranded. The oligonucleotide can be labeled with a single fluorescent label or with a fluorescent pair (donor and acceptor) on a single-strand of DNA or RNA. The choice of single or double label will depend on the efficiency of the enzyme employed in the method of the invention. For example, a single fluorescent label can best be used with an efficient enzyme
10 such as a restriction endonuclease.

In the method of the invention, there is no limitation on the length of the oligonucleotide substrate, so long as the fluorescent probe is labeled 6-7 nucleotides away from the enzyme cleavage site.

15 The term "fluorescent label" or "fluorophore" as used herein refers to a substance or portion thereof that is capable of exhibiting fluorescence in the detectable range. Examples of fluorophores that can be used according to the invention include fluorescein isothiocyanate, fluorescein amine, eosin, rhodamine, dansyl, and umbelliferone. Other fluorescent labels will be known to the skilled artisan.

20 Some general guidance for designing sensitive fluorescent labelled polynucleotide probes can be found in Heller and Jablonski's U.S. Patent 4,996,143. This patent discusses the parameters that should be considered when designing fluorescent probes, such as the spacing of the fluorescent moieties (i.e., when a pair of fluorescent labels is utilized in the present
25 method), and the length of the linker arms connecting the fluorescent moieties to the base units of the oligonucleotide. The term "linker arm" as used herein is defined as the distance in Angstroms from the purine or pyrimidine base to which the inner end is connected to the fluorophore at its outer end.

30 Preferably, in the method of the present invention, the donor and acceptor fluorophores should be attached to the oligonucleotide at positions which give them a relative separation of zero to twenty base units. The

-11-

preferred separation is from zero to seven base units. The preferred length of the linker arm is a 12 carbon chain.

The term "cleavage that is enzyme-mediated" refers to cleavage of DNA or RNA that is catalyzed by such enzymes as DNases, RNases, helicases, exonucleases, restriction endonucleases, or retroviral integrases. Other enzymes that effect nucleic acid cleavage will be known to the skilled artisan and can be employed in the practice of the present invention. A general review of these enzymes can be found in Chapter 5 of Sambrook *et al, supra*.

Fluorescently labeled oligonucleotides and DNA fragments have been utilized in nucleic acid research, with applications that include DNA hybridization, automated DNA sequencing, fluorescence anisotropy, and resonance energy transfer studies. Past concerns with fluorescent-labeled DNA arose from interactions between fluorophores and DNA that result in quenched fluorescence. This quenching phenomenon is most problematic in FRET studies because quenching of the donor fluorescence could result from either resonance energy transfer or non-transfer effects.

In the first embodiment of the method of the present invention, cleavage of a single fluorescently labeled oligonucleotide substrate bearing the BamHI restriction site, was examined using the enzyme DNase I and the restriction endonuclease BamHI. Relief of non-transfer quenching of a 14-mer FITC-labeled oligonucleotide was characterized with both steady-state and time-resolved fluorescence techniques. The FITC-labeled single-strand was best fit by a triexponential decay with lifetimes of 0.5, 2.7, and 4.2 ns. The 4.2 ns component was found to contribute more than 80% of the total steady-state intensity. Upon annealing with an unmodified complementary strand, the contribution from the 4.2 ns component was significantly decreased, resulting in two-fold quenching of total fluorescence. The inventors reasoned that this quenching phenomenon should be a reversible process and, therefore, could be employed to study the numerous strand separation processes in molecular biology. The results demonstrated that the quenched fluorescence is totally

-12-

recovered upon cleavage (compared to that of the single-strand). The extent of cleavage measured by fluorescence was confirmed by non-denaturing polyacrylamide gel electrophoresis analysis. This fluorescence "dequenching" technique can be used to quantify the kinetics of other restriction endonucleases, as well as other DNA strand separation and cleavage processes known to those skilled in molecular biology. It is also expected that the converse principal (decreased fluorescence) can be applied to methods in molecular biology involving nucleic acid ligation, as opposed to cleavage.

In the method of the present invention, chemical modifications of oligonucleotides and DNA fragments are easily accomplished by known methods using nucleotide analogs (Ruth *et al.*, *DNA* 4:93 (1985); Telser *et al.*, *J. Am. Chem. Soc.* 111:6966 (1989)) and DNA synthesis reagents. Phosphoramidites containing aliphatic primary amines may be introduced into the oligonucleotides at desired positions through automated DNA synthesizers. For example, Aminolink 2 (Applied Biosystems) and amino-modified-dT (Glen Research) can be used to introduce an aliphatic primary amine with a six-carbon linker at the 5'-end of oligonucleotides. This amine can react with a variety of substrates such as biotin (Chu and Orgel, *DNA* 4:327-331 (1985); Chollet *et al.*, *Nucleic Acids Res.* 13:1529-1541 (1985)), fluorescent dyes (Cardullo *et al.*, *supra*; Murchie *et al.*, *Nature* 341:763-766 (1989); Clegg *et al.*, *supra*; Cooper and Hagerman, *supra*), EDTA (Dreyer and Dervan, *Proc. Natl. Acad. Sci. USA* 82:968-972 (1985)), or alkaline phosphatase (Jablonski *et al.*, *Nucleic Acids Res.* 14:6115-6128 (1986)) to form oligonucleotide conjugates. Applications of these modified oligonucleotides include: (i) nonradiolabeled hybridization probes (Chu and Orgel, *supra*; Chollet *et al.*, *supra*; Jablonski *et al.*, *supra*; Connolly, B.A., *Nucleic Acids Res.* 15:3131-3137 (1987)); (ii) sequence-specific cleavage of DNA (Dreyer and Dervan, *supra*); (iii) automated DNA sequencing (Brumbaugh *et al.*, *Proc. Natl. Acad. Sci. USA* 85:5610 (1988)); and (iv) affinity chromatography (Ruth *et al.*, *Fed. Proc.* 44:1622 (1985)). Moreover, this approach allows application of fluorescence spectroscopic techniques to structural studies of

-13-

nucleic acids (Murchie *et al.*, *supra*; Clegg *et al.*, *supra*; Cooper and Hagerman, *supra*).

In the method of the present invention, the modified oligonucleotides containing primary amines are derivatized with fluorescent probes, as discussed above, and are used in a continuous assay to detect nucleic acid cleavage *in vitro*, by monitoring increases in fluorescence.

The second embodiment of this invention is directed to the application of this fluorescent assay to other less efficient enzymes involved in nucleic acid cleavage, such as retroviral integrase proteins (IN).

Integration of viral DNA into the host chromosome is an essential step in the life cycle of retroviruses. The integration reaction is known to be catalyzed by the integrase protein, which is encoded by the retroviral *pol* gene. Integration requires a particular sequence at the ends of the linear double-stranded viral DNA that is synthesized by reverse transcription from the viral RNA genome in the infected cells (Donehower *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6461-6465 (1984); Scgwartzberg *et al.*, *Cell* 37:1043-1052 (1984); Panganiban *et al.*, *Proc. Natl. Acad. Sci. USA* 81:7885-7889 (1984); Hippermeyer *et al.*, *Virology* 137:358-370 (1984); Clavel *et al.*, *J. Virol.* 63:1455-1459 (1989); Grandgenett *et al.*, *Cell* 60:3-4 (1990); Varmus *et al.*, "Retrovirus," in *Mobile DNA*, Berg, D.E. and Howe, M.M., eds., American Society for Microbiology, Washington, D.C. (1989), pp. 53-108).

Initially, human immunodeficiency virus type 1 (HIV-1) integrase recognizes the specific DNA sequence, -CAGT at the 3' end of the viral DNA and removes two bases (GT-3') from each 3' end. Subsequently, the 3' ends expose the CA_{OH} and become joined to the 5' ends of target chromosomal DNA strands at the site of integration (Fujiwara *et al.*, *Cell* 54:497-504 (1988); Brown *et al.*, *Proc. Natl. Acad. Sci. USA* 86:2525-2529 (1989); Fujiwara *et al.*, *Proc. Natl. Acad. Sci. USA* 86:3065-3069 (1989)). The cleavage and joining processes seem to be a coupled event. Evidence of this is that there is no requirement of an exogenous energy source (Craigie *et al.*, *Cell* 62:829-837 (1990); Katz *et al.*, *Proc. Natl. Acad. Sci. USA* 89:6741-6745

(1992)). As a result of the IN mediated cleavage-ligation reaction, a gapped recombination intermediate is produced. The completion of integration must require a gap repair process, which is presumably mediated by host enzymes.

5 *In vitro* studies with short synthetic oligonucleotides corresponding to either U5 or U3 ends of viral DNA have demonstrated that the integration reaction (3'-processing and strand transfer) can be catalyzed by the purified integrase alone (Craigie *et al.*, *Cell* 62:829-837 (1990); Katz *et al.*, *Proc. Natl. Acad. Sci. USA* 89:6741-6745 (1992); Sherman *et al.*, *Proc. Natl. Acad. Sci. USA* 87:5119-5123 (1990); Katzman *et al.*, *J. Virol.* 63:5319-5327 (1989); Bushman *et al.*, *Proc. Natl. Acad. Sci. USA* 88:1339-1343 (1991)).
10 The only requirements for this *in vitro* reaction are a linear DNA substrate, integrase, and a divalent metal cation (either Mg^{2+} or Mn^{2+}). Recent mechanistic studies of the 3' processing and strand transfer reaction indicate that the strand transfer process is a one-step reaction. In one concerted
15 reaction, a phosphodiester bond in the target DNA is cleaved and a new bond (between viral DNA and target DNA) is formed (Engelman *et al.*, *Cell* 67:1211-1221 (1991)). This conclusion was made by examining the stereochemical course of the reactions catalyzed by HIV-IN. The chirality of the phosphothioate in the reaction products was determined by incorporating
20 phosphothioate of known chirality in substrate DNAs. Further *in vitro* studies with an oligonucleotide substrate that mimics the recombination intermediate have shown that integrase can also promote a reverse reaction termed disintegration (Chow *et al.*, *Science* 255:723-726 (1992). The activities of integrase are therefore characterized as donor cutting, strand transfer,
25 disintegration, and integration site selection.

 In this second embodiment of the present invention, a continuous spectroscopic assay system was developed to characterize retroviral integrase mediated nucleic acid cleavage reactions. In this embodiment, the assay preferably employs FRET, and, consequently, a pair of fluorescent labels
30 (fluorescent donor and acceptor). This assay was developed to combine the additive effects of the quenching of donor fluorescence due to probe-strand

-15-

interactions and the quenching of donor fluorescence due to energy transfer. Such an assay would therefore display a larger recovery of the donor fluorescence.

In a preferred embodiment, a double-stranded oligonucleotide representing the U5 or U3 end of HIV-DNA, is site-specifically labeled with a pair of extrinsic fluorophores, fluorescein isothiocyanate (FITC), and eosin isothiocyanate (EITC) at the 3' end and the 5' end of the substrate DNA, respectively. A variety of fluorophores can be covalently attached to the oligonucleotide substrate, which has been modified with nucleotide analogs containing primary amines. Generally, the donor and acceptor fluorescent moieties should be selected so that the emission spectra of the donor moiety overlaps the excitation spectrum of the acceptor moiety to produce efficient non-radiative energy transfer therebetween. Such exemplary fluorophore pairs include fluorescein (fluorescence donor) and eosin (fluorescence acceptor), and fluorescein (fluorescence donor) and tetramethylrhodamine isothiocyanate (fluorescence acceptor). Other suitable donor-acceptor combinations that can be utilized in the method of the present invention will be known to the skilled artisan. Further guidance on appropriate fluorescent label combinations can be found in U.S. Patent 4,996,143, columns 5-6.

The two different fluorophores (fluorescent donor and fluorescent acceptor) can easily be manipulated to be in close proximity. This can be accomplished either by introducing them to the same strand of DNA or by modifying two separate strands followed by annealing. The donor fluorescence will be quenched as energy is transferred to the acceptor. Upon physical separation of these two fluorophores by enzymatic cleavage, the quenched donor fluorescence will be recovered as FRET is lost. Since HIV integrase will recognize the 3'-TGAC and cleave the 3'-TG exposing the CA_{OH}-3' while the 5'-end complementary sequence, ACTG, will not be cleaved by HIV-IN, the fluorescein group attached to the 3'-end would be removed from the DNA substrate, resulting in a loss of energy transfer between fluorescein and eosin. Therefore, enzymatic cleavage of the substrate

-16-

will result in enhancement of the quenched fluorescence signal. Hence, monitoring these fluorescence changes detects DNA cleavage reactions of HIV-IN.

One advantage of utilizing the enhancement of fluorescence is that it will serve to amplify the observed cleavage signal. For example, if the fully recovered donor fluorescence is 10 fold higher than the quenched fluorescence, 10% DNA cleavage will still exhibit a two-fold increase in the donor fluorescence. Since HIV-IN usually displays incomplete cleavage activity, the amplification of the signal is an important feature of this fluorescence system. FRET further allows one to measure the separate processes that are involved in DNA integration and repair systems. Fluorescence offers numerous measurable parameters that with various systems can be adapted to detect fluorescent bands upon gel electrophoresis capable of detecting as little as 4 picograms of double-stranded DNA (Glazer *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3851 (1990)) or to monitor numerous samples in a 96-well fluorescence microplate reader. More importantly, a fluorescence assay is continuous, making it possible to obtain precise kinetic parameters for mechanistic studies of integrase proteins. A further advantage is that the degree of donor fluorescence quenching is easily manipulated in a range of 10 to 40-fold, depending on the pair and distance between the pair of fluorophores. (Matayoshi *et al.*, *Science* 247:954-957 (1990)). This assay system would also provide rapid and sensitive measurements on the effectiveness of specific inhibitors on HIV-IN or other well-studied retroviral integrase proteins, such as the integrase protein of Moloney Murine Leukemia Virus (MoMLV) (Craigie *et al.*, *Cell* 62:829-837 (1990)) and avian sarcoma-leukosis virus (ASLV) (Terry *et al.*, *J. Virol.* 62:2358-2365 (1988)).

The described fluorometric assay for detecting nucleic acid cleavage can also be utilized to improve the efficiency and detection signal of a number of well-known procedures for amplifying or detecting a specific DNA or RNA sequence, such as polymerase chain reaction (PCR), ligase chain reaction, and catalytic hybridization amplification or "cycling probe" technology.

-17-

Having now generally described this invention, the same will be better understood by reference to one or more specific examples. These examples are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

5

Example 1

Fluorometric Assay for Detecting Nucleic Acid Cleavage by the Restriction Enzyme, BamHI

Materials

10 FITC and 5-carboxyfluorescein were obtained from Molecular Probes (Eugene, OR). The *BamHI* restriction enzyme was purchased from New England Biolab. Aminolink 2 and reagents for oligonucleotide synthesis were obtained from Applied Biosystems. DNase I was obtained from Worthington Enzymes.

Methods

15 ***Preparation of fluorescent-labeled oligonucleotides:*** Aminolink 2 is a commercially available modified base that can be directly introduced into oligonucleotides with a DNA synthesizer. This reagent introduces an aliphatic primary amine at the 5' ends of oligonucleotides. Accordingly, a 14-mer aminolink oligonucleotide, 5'-NH₂-CCCCGGATCCACCC-3' (SEQ ID NO:1),
20 containing the *BamHI* restriction site GGATC, and its complementary strand
without aminolink, 3'-GGGGCCTAGGTGGG-5' (SEQ ID NO:2), were
synthesized using an Applied Biosystems 380B DNA synthesizer. The oligonucleotides were purified by an HPLC Zorbak bio series oligo column (DuPont). The aminolink oligonucleotide was then derivatized with FITC in
25 100 mM NaHCO₃/Na₂CO₃ buffer, pH 9.0. The FITC was initially dissolved in DMF and then added to the oligonucleotide solution (20% v/v). Excess

dye was removed by filtration of the reaction mixture through a Sephadex G-25 column (DNA grade). The resulting sample and its complementary strand were then electrophoresed on denaturing (7M urea) 20% polyacrylamide gels to further purify the oligonucleotides and to remove any residual free dyes. The appropriate oligonucleotide bands were sliced from the gels and electroeluted using the S&S ELUTRAP Electro-Separation System (Schleicher & Schuell).

Spectroscopic Measurements: Absorbance and absorption spectra were measured with a Hewlett-Packard 8450A diode array spectrophotometer. Using the extinction coefficient of fluorescein (E-78,000) at 492 nm, the concentration and total moles of fluorescein were calculated (Chen and Scott, *supra*). The amount of fluorescein conjugated to the oligonucleotide was estimated by subtracting the moles of fluorescein multiplied by the extinction coefficient of FITC at 260 nm, 23,000 M⁻¹ cm⁻¹, from the total absorbance at 260 nm. The remaining AU₂₆₀ should be proportional to the DNA content. Steady-state fluorescence spectra and intensity were recorded with an SLM 8000 spectrophotofluorometer with 10-mm Glan-Thompson polarizers. Fluorescence emission measurements were performed under "magic angle" emission conditions (Spencer and Weber, *J. Chem. Phys.* 52:1654-1663 (1970)). A cuvette with a 3-mm excitation path length was used for all experiments. The absorbance of all fluorescence samples was less than 0.1 at the wavelength of excitation to avoid inner-filter effects. The temperatures of the samples were regulated with a Neslab Instruments, Inc., T.E.Q. temperature controller and a PBC4 bath cooler.

Time-resolved fluorescence was measured on a time correlated single-photon counting instrument. Excitation was accomplished by a synchronously pumped, mode-locked, cavity dumped dye laser (Spectra-Physics 2045E argon/3520 dye) capable of producing 10-ps (fwhm) pulses at a frequency of 4 MHz, which are frequency doubled to UV. Time-resolved experiments were also performed under "magic angle" conditions. The excitation wavelength was 310 nm, and the emission wavelengths were selected via a

-19-

stepper motor-driven monochromator. 1,8-ANS and 5-carboxyfluorescein were used as fluorescence standards to verify proper functioning of the instrument, and to correct for the wavelength-dependent transmit time of the Hamamatsu R955 photomultiplier. All fluorescence measurements were performed with the samples in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, and 0.1 M NaCl, which is the optimal buffer condition for *Bam*HI cleavage reactions.

The extent of cleavage by *Bam*HI was calculated from the fluorescence data utilizing the following equation:

$$[DNA]_c = \frac{F_t - F_0}{F_\infty - F_0} \times [DNA]_i \quad (\text{Equation 1})$$

where $[DNA]_c$ is the concentration of cleaved DNA, F_t is the fluorescence intensity at time, t , F_∞ is the fluorescence intensity at the plateau, F_0 is the initial fluorescence intensity, and $[DNA]_i$ is the initial concentration of DNA.

Results and Discussion

The quenching of fluorophores attached to oligonucleotides via Aminolink 2 (six carbon chain linker) has been previously described (Murchie *et al.*, *supra*; Clegg *et al.*, *supra*; Cooper and Hagerman, *supra*). This quenching was attributed to direct interactions between the probes and the DNA. To further characterize this quenching behavior, a 14-mer *Bam*HI target oligonucleotide was synthesized that includes a primary amino group introduced at the 5'-end via Aminolink 2. The resulting amino group was then derivatized with FITC in 100 mM NaHCO₃/Na₂CO₃, pH 9.0, and 20% DMF.

Both steady-state and time-resolved fluorescence measurements were employed. Initially, the steady-state fluorescence intensity of the FITC-labeled single-strand and the double-strand annealed to its nonlabeled complementary strand were compared. To a fixed concentration of FITC-

labeled oligonucleotides (0.137 pmol), varying concentrations of the unlabeled complementary strands were added and annealed in 0.5 mL of 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, and 0.1 M NaCl. Changes in the emission spectra of fluorescein were monitored with an excitation wavelength of 490 nm at 37°C. Figure 1 shows the progressive quenching of fluorescence due to the increasing degrees of annealing. Approximately two fold quenching of fluorescein intensity was observed when the labeled oligonucleotide was annealed to equal concentrations of nonlabeled sample. Further quenching of fluorescence was not observed when a 1.5 M excess concentration of unlabeled complementary strand was added, indicating that changes in fluorescein intensity were primarily due to the degree of annealing. The annealed complex also displayed blue-shifted emission spectra. To confirm that the observed quenching is associated with annealing and to examine if this process is reversible, an excess concentration of DNase I was added. As shown in Figure 1, the initial fluorescence intensity was fully recovered (compared to that of the single-strand).

The aforementioned quenching was characterized with time-resolved fluorescence studies. A series of decay curves was collected as a function of emission wavelength from both the single-strand and annealed samples. The decay curves were simultaneously analyzed using a global analysis procedure (Knutson *et al.*, *Chem. Phys. Lett.* 102:501-504 (1983); Beechem *et al.*, *Anal. Instrum.* 14:379-402 (1985)). The best fit of the data was achieved by triexponential decay analysis. The recovered decay components were 0.5, 2.7, and 4.2 ns (Figures 2A and 2B). The single-strand sample exhibits a predominant decay component of 4.2 ns (Figure 2A). Upon annealing, this component was severely decreased (Figure 2B). The decay-associated spectra of the two minor decay components were blue-shifted compared to the decay-associated spectrum of the 4.2 ns component. Hence, pure *static* quenching (and/or loss of the corresponding absorbing species in the ground state) of the 4.2 ns component results both in loss of steady-state intensity and in the emergent blue-shift of the steady-state spectra. These results confirm previous

-21-

observations regarding labeled oligonucleotides (Clegg *et al.*, *supra*; Cooper and Hagerman, *supra*).

Since DNase I reversed the quenching of the double-strand DNA fragment at 37°C, the DNA cleavage process of the restriction endonuclease *Bam*HI was examined by monitoring changes in the fluorescence intensity. The Type II restriction endonuclease *Bam*HI recognizes the duplex symmetrical sequence 5'-GGATCC-3' (Wilson and Young, *J. Mol. Biol.* 97:123-125 (1975)). Accordingly, oligonucleotide samples containing this recognition sequence were synthesized as shown below:

10 5'-FITC-NH-CCCCGGATCCACCC-3' (SEQ ID NO:1)
 3'-GGGGCCTAGGTGGG-5' (SEQ ID NO:2)

To ensure complete annealing of the ends of the oligonucleotides, consecutive cytosines and guanines were designed at both ends. In the presence of Mg²⁺, the enzyme catalyzes double-strand cleavage between the guanines, generating 15 5'-phosphoryl and 3'-hydroxyl staggered termini. Cleavage by the *Bam*HI restriction endonuclease results in the fragments:

5'-FITC-NH-CCCCG + GATCCACCC-3'
 3'-GGGGCCTAG GTGGG-5'

The cleaved products will be two fragments of 5 base pairs each; these should 20 have relatively low melting temperatures. Dissociation of the two strands should result in total recovery of fluorescence intensity. Figures 3A and 3B depict the changes in fluorescence intensity due to the *Bam*HI restriction endonuclease. The labeled oligonucleotide (0.208 nmol) was annealed with a 1.5 M excess concentration of unlabeled complementary strand and then 25 digested with 20 units of *Bam*HI in a 400 µl solution containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, and 0.1 M NaCl. The cleavage experiments were monitored at both 25°C (Figure 3A) and the optimal temperature for

-22-

*Bam*HI cleavage, 37°C, (Figure 3B) to determine any differences in the extent of fluorescence recovery. The emission spectra of the cleaved samples were recorded with an excitation wavelength of 490 nm after the fluorescence changes at an emission wavelength of 520 nm had reached a plateau. As expected, both samples resulted in increased fluorescence intensity compared to the uncleaved DNA at both temperatures. To compare the differences observed in the extent of fluorescence recovery, the intensities of the samples at both temperatures were peak normalized according to the values of uncleaved substrates. It was also observed that changes in fluorescence intensity occurred *only* with annealed samples in the presence of Mg²⁺. No changes in fluorescence were observed with the single-strand alone, annealed samples in the presence of EDTA, or after the methylation of the substrate by *Bam*HI methyltransferase, indicating that the observed fluorescence changes are the direct result of cleavage by *Bam*HI.

It was noted that the changes in fluorescence intensity were significantly greater at 37°C than at 25°C. This discrepancy could be accounted for by the incomplete dissociation of the 5-mer products to their single-strand components at 25°C. Using the computer program OLIGO (Rychlik and Rhoads, *Nucleic Acids Res.* 17:8543-8551 (1989)), the predicted T_m of the 5-mer sequence by percentage GC methods is -12.5°C, whereas the predicted T_m using (AT * 2°C + GC * 4°C) (Suggs *et al.*, in *ICN-UCLA Symposium on Development of Biology Using Purified Genes*, Brown (ed.), Academic Press, Inc., New York (1981), pp. 683-693) is 20°C. These formulae give an estimated temperature at which 50% of the oligonucleotide duplexes are dissociated. Therefore, it would be expected from the latter formula that all of the 5-mer sequences would not dissociate to their respective single-strands at room temperature (25°C), whereas all of the cleaved products would dissociate at 37°C.

Time-resolved studies were also performed with the cleaved products of the FITC-labeled DNA substrates at 25°C. Once again, a series of decay curves was collected as a function of emission wavelength (Figure 4). The

cleaved sample exhibits the same three decay components found for annealed and single-stranded samples. As expected, the amplitude associated with the 4.2 ns component had increased compared to that of the uncleaved (annealed) sample. However, the 4.2 ns component did not fully recover to the original amplitude of the single-strand sample. Since these decay measurements were performed at 25°C, the prior (incomplete dissociation) effects observed in Figure 3A could account for the lack of complete recovery.

Both the results above and the DNase I data (Figure 1) demonstrate that full recovery of fluorescence intensity is observed at 37°C. However, examining the possibility of partial dissociation of the double-stranded DNA substrate was also deemed important. As expected, the single-stranded sample showed a decrease in intensity as temperature increased due to an increased rate of internal conversion, which decreases the observed fluorescence. In contrast, the fluorescence intensity of the annealed sample increased with temperature.

To correct for the direct temperature effects on fluorescence intensity, the intensity *ratio* of the double-stranded and single-stranded samples was plotted as a function of temperature (Figure 5). Subsequently, the apparent melting temperature of the annealed double-stranded sample was obtained. The predicted T_m of the annealed sample using $AT * 2^\circ C + GC * 4^\circ C$ is 50°C, whereas the predicted T_m using percentage GC methods is 65.5°C. These results suggest a T_m for the double-stranded sample of approximately 65°C, which is in better agreement with the percentage GC method. It should be emphasized that there are no significant changes in fluorescence intensity observed in the temperature range from 20°C to 45°C, ensuring that the optimal assay conditions for *BamHI* are in the thermally stable region for the annealed substrate.

Utilizing this information, the kinetics of DNA cleavage by *BamHI* was subsequently studied at 37°C. Differing concentrations of the enzyme (10, 20, 40, and 60 units) were added to a fixed concentration of substrate, 0.208 nmol, in 420 μ l of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 0.1 M

NaCl. Figure 6 shows how the apparent rates of cleavage increase as a function of enzyme concentration. These rates are the composite of two processes: cleavage of the substrate by *Bam*HI and the subsequent dissociation of the cleaved products into their single-strand components. The rate-limiting step of the overall process is cleavage by *Bam*HI. For reasons discussed previously, the rate of dissociation will not contribute to the observed rates of cleavage because dissociation of the 5-mer sequences should be relatively fast at 37°C. Initial velocities were determined from the linear portions of the curves (Figure 6, inset). The plot of the initial velocities versus enzyme concentrations was linear, suggesting that the rate of cleavage was first order with respect to *Bam*HI concentration, and that the rates observed were due to cleavage of the substrate.

Cleavage reactions were also examined as a function of substrate concentration with a fixed concentration of *Bam*HI (10 units in 100 μ l total reaction volume). The reactions were initiated by the addition of *Bam*HI and stopped by the addition of 25 mM EDTA 11 minutes after the enzyme was added. The reaction mixtures were subjected to native 20% polyacrylamide gel analysis. Since the DNA substrate was labeled with FITC, a photograph of the gel was taken under ultraviolet illumination (using a Kodak green filter) without staining the gel with ethidium bromide. Therefore, only the fluorescent-labeled DNA fragments were visualized, resulting in two DNA bands: a substrate band and a product band (5'-FITC-NH-CCCCG). A direct comparison was made between the data obtained from the fluorometric assay and the photographic data obtained from gel electrophoretic analysis. The kinetics of *Bam*HI cleavage reactions as a function of DNA substrate concentration measured by fluorometric analysis are illustrated in Figure 7A. In the presence of 25 mM EDTA, no changes in fluorescence intensity were observed (curve E), indicating that no cleavage of the substrate occurred. The fluorescence intensity at low concentrations of substrate (0.21 μ M, curve A) was observed to plateau approximately 6 minutes after the initiation of reaction. This indicates that the reaction reached completion; i.e., there was

-25-

no more substrate available for cleavage. This conclusion was further confirmed by data obtained from gel electrophoresis analysis, which showed 100% conversion of substrate to the product (Figure 7B, lane A). Similarly, the reaction at 0.36 μ M substrate reached near completion as shown by both the fluorescence (curve B, Figure 7A) and the gel data (lane B, Figure 7B). Higher DNA substrate concentrations, 0.72 μ M (curve C, Figure 7A) and 1.08 μ M (curve D, Figure 7A), yielded incomplete cleavage reactions (observed by changes in fluorescence intensity). The time frame depicted here does not allow the reaction to go to completion at high substrate concentrations. Curve C, however, shows higher net fluorescence changes than those observed for curve D, indicating that more cleavage products should be observed for C. It appears that the initial velocity of curve D is also slower than that of curve C. These results may be due to pipeting error since the reaction is initiated by the addition of 0.5 μ l of *Bam*HI (20 units/ μ l) that is stored in 50% glycerol. As expected, the PAGE data (Figure 7B) show higher product formation in lane C compared to that of lane D.

The results of both sets of data are summarized in Table 1. The fractions of uncleaved and cleaved DNA were calculated from the fluorescence data utilizing equation 1 (see Methods). Fractions of uncleaved and cleaved DNA were also estimated from a photograph taken from the polyacrylamide gel. Utilizing a Hewlett Packard ScanJet IIP and the densitometry program Scan Analysis 68000 (BioSoft), the peaks obtained from each of the bands (substrate and products) were integrated and ratioed to obtain the relative amount of cleavage. As illustrated in Table 1, the data obtained from the fluorescence measurements correlate well with those obtained from the gel, confirming the viability of the kinetic assay.

-26-

Table 1 Summary of Percentage Cleavage of FITC-labeled DNA Substrates by <i>Bam</i>HI, Estimated by Both Fluorometric and PAGE Analysis			
Curves	[DNA] μM	% Cleavage Fluorescence Assay	% Cleavage PAGE
A	0.21	100	100
B	0.36	91.9	92.3
C	0.72	59.5	60.6
D	1.08	32.2	30.1
E	0.18	0	0

In summary, using the *Bam*HI restriction endonuclease as an exemplary system, the results presented in this Example demonstrate the feasibility of applying the fluorescence "dequenching" phenomenon to kinetic studies of other restriction endonucleases. The fluorescence assay presented herein provides an easy and rapid method for acquiring high data density essential for precise kinetic studies (e.g., quantifying sequence discrimination by base-analog substitutions). It should also be possible to employ this approach to develop a coupled assay for sequence-specific DNA methylase activity. A continuous fluorometric assay is highly advantageous over the conventional discontinuous gel electrophoresis assay systems. Kinetic data obtained from this continuous system based on fluorescence dequenching may significantly improve studies of enzymatic reactions in molecular biology, and is a viable alternative to FRET in studying cleavage and strand separation processes in molecular biology.

Example 2

Fluorometric Assay for Detecting Nucleic Acid Cleavage Mediated by HIV-integrase

Materials

5 5-Amino (12)-2'-deoxyuridine β -cyanoethyl phosphoramidite was obtained from Molecular Biosystems, Inc. FITC, EITC, 5-carboxyfluorescein, and 1,8-ANS were purchased from Molecular Probes. [γ -³²P]ATP was obtained from ICN. T4 polynucleotide kinase was obtained from New England Biolabs. Ni²⁺-charged metal chelating resin was
10 purchased from Novagen.

Methods

Preparation of HIV-IN protein: The wild-type HIV-integrase protein was obtained from Dr. Robert Craigie (Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda,
15 Maryland). The protein was overexpressed in *Escherichia coli* and purified according to previously-described procedures. (Sherman and Fyfe, *Proc. Natl. Acad. Sci. USA* 87:5119-5123 (1990)).

Preparation of Oligonucleotides and Fluorescent Labeled DNA Substrate for HIV-IN: Oligonucleotides containing the terminal sequence of
20 HIV-1 DNA were synthesized using a DNA synthesizer and annealed to form the following substrates:

-27.1-

Substrate 1:

F-D1/T1: 5'-TGAGTACCCGCTGTGGAAAATCTCTAGCAGGGNCTATGGCGTCCCTCTG (SEQ ID NO:3)
E-D2: 3'-ACTCATGGGCACACCTTTTAGAGATCGTCAN (SEQ ID NO:4)

Substrate 2:

F-D1/T1: 5'-TGAGTACCCGCTGTGGAAAATCTCTAGCAGGGNCTATGGCGTCCCTCTG (SEQ ID NO:3)
D3: 3'-ACTCATGGGCACACCTTTTAGAGATCGTCCAGATACCGCAGGGGAGAC (SEQ ID NO:5)

Bold letters are the sequences of the U5 end of the HIV-1 DNA (See Smith *et al.*, *J. Virol* 64:6286-6290 (1990)) and N depicts the position of the nucleotide analog that contains an aliphatic primary amine. F indicates FITC, E indicates EITC, D indicates donor strand, and T indicates target strand. D3 does not have a fluorescent label. The oligonucleotides were purified by an HPLC Zorbak bio series oligo column (Du Pont).

5-Amino (12)-2'-deoxyuridine β -cyanoethyl phosphoramidite is a commercially available modified base that can be directly introduced into oligonucleotides with a DNA synthesizer. This reagent substitutes for dTTP and introduces an aliphatic primary amine at the specified positions in the oligonucleotides. The D1/T1 and D2 oligonucleotides containing this nucleotide analog were then derivatized with a pair of fluorescent FITC and EITC dyes, in 100 mM NaHCO₃/Na₂CO₃ buffer pH 9.0, respectively. Excess dye was removed by filtration of the reaction mixture through a Sephadex G-25 column (DNA grade). The resulting samples were then electrophoresed on denaturing (7M urea) 20% polyacrylamide gels to purify further the oligonucleotides and to remove any residual free dyes. The appropriate oligonucleotide bands were sliced from the gels and electroeluted using the S&S ELUTRAP Electro-Separation System from Schleicher & Schuell.

Spectroscopic Measurements: Absorbance and absorption spectra were measured with a Hewlett-Packard 8450A diode array spectrophotometer. Steady-state fluorescence spectra and intensity were recorded with an SLM 8000 spectrophotofluorometer with 10-mm Glan-Thompson polarizers. Fluorescence emission measurements were performed under "magic angle" emission conditions (Spencer and Weber, *J. Chem. Phys.* 52:1654-1663 (1970)). A cuvette with a 3 mm excitation path length was used for all experiments. The absorbance of all fluorescence samples was less than 0.1

at the wavelength of excitation to avoid inner filter effects. The temperatures of the samples were regulated with a Neslab Instruments, Inc. T.E.Q. temperature controller and a PBC4 bath cooler.

5 Time-resolved fluorescence was measured on a time correlated single-photon counting instrument. Excitation was accomplished by a synchronously pumped, mode-locked, cavity dumped dye laser (Spectra-Physics 2045E argon/3520 dye) capable of producing 10 ps (fwhm) pulses at a frequency of 4 MHz, which are then frequency doubled to UV. Time-resolved experiments were also performed under "magic angle" conditions. The excitation
10 wavelength was 310 nm, and the emission wavelengths were selected via a stepper motor-driven monochromator. 1,8-ANS and 5-carboxyfluorescein were used as fluorescence standards to verify proper functioning of the instrument and to correct for the wavelength-dependent transmit time of the Hamamatsu R955 photomultiplier.

15 *Radioactive DNA cleavage reaction:* One microgram of the appropriate oligonucleotide was ^{32}P -labeled at the 5' termini by use of T4 polynucleotide kinase and 25 μCi of $[\gamma\text{-}^{32}\text{P}]\text{adenosine 5'-triphosphate}$. The labeled oligonucleotide was annealed with 3 fold molar excess of unlabeled complementary strand(s) in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and
20 0.1 M NaCl. All the reaction mixtures for the IN protein mediated cleavage reactions contained 25 mM HEPES, pH 7.5, 2.5 mM DTT, 60 mM NaCl, 5% glycerol (v/v), 7.5 mM Mg^{2+} , ^{32}P labeled fluorescent substrates, and HIV-1 IN in a total volume of 15 μl . The reactions were initiated by addition of IN protein, and the reaction mixtures were incubated up to 90 minutes at
25 37°C. The reactions were stopped by the addition of an equal volume of stop solution (95% formamide, 30 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue) to each reaction and boiled for 5 minutes. Ten μl of each reaction mixture was electrophoresed on a 7 M urea denaturing 15% polyacrylamide sequencing gel and reaction products analyzed by
30 autoradiographic densitometry.

Results and Discussion

It has recently been demonstrated that the endonuclease activity exhibited by HIV-IN could be altered in terms of its efficiency of cleavage and metal requirement depending on the length and structure of substrates (Lee *et al.*, *J. Biol. Chem.*, submitted 1994). The longer substrates that were utilized contained a 49-mer hybrid strand consisting of 19 base pairs corresponding to the sequence at the U5 end of the HIV-1 DNA, with 9 additional random base pairs at the 5' end of the donor strand, and 21 random nucleotides as the target sequence at the 3' end. This longer substrate displayed Mg^{2+} dependent endonucleolytic cleavage. When the 9 random base pairs were substituted with the endogenous sequences corresponding to the U5 end of the HIV-1 DNA, the cleavage pattern was identical to the original 49-mer hybrid with a preference for Mg^{2+} . Therefore, the inventors concluded that increases in the length of both donor and target sequences in the hybrid strand result in the enhancement of activation by Mg^{2+} over Mn^{2+} , bringing the *in vivo* and *in vitro* reaction conditions into agreement.

In this Example, the Mg^{2+} -dependent endonuclease activity of HIV-IN was further characterized utilizing fluorescence resonance energy transfer. The 49-mer hybrid strand and its partial complementary strand were modified to contain a fluorescence donor and acceptor. The sequence and the cleavage reaction of the fluorogenic substrate 1 are shown below:

-30.1-

FITC

F-D1/T1: 5' -TGAGTACCCGCTGTGGAAAATCTCTAGCAGGGNCTATGGCGTCCCTCTG (SEQ ID NO:3)
E-D2: 3' -ACTCATGGGCACACCTTTTAGAGATCGTCAN-EITC (SEQ ID NO:4)

+ HIV-IN

FITC

5' -TGAGTACCCGCTGTGGAAAATCTCTAGCA (SEQ ID NO:5) + GGGNCTATGGCGTCCCTCTG (SEQ ID NO:6)
3' -ACTCATGGGCACACCTTTTAGAGATCGTCAN-EITC (SEQ ID NO:4)

Both FITC and EITC were covalently labeled to a primary amine group of a nucleotide analog (N), 5-amino (12)-2'-deoxyuridine β -cyanoethyl phosphoramidite. This analog is introduced at specified positions in the oligonucleotides by substituting for dTTP via a DNA synthesizer. Significant resonance energy transfer from fluorescein to eosin (calculated Förster distance of 54 Å, Carraway *et al.*, *J. Biol. Chem.* 264:8699-8707 (1989)) was expected owing to the strong spectral overlap between the emission spectrum of fluorescein ($\lambda_{\text{MAX}} = 520$ nm) and the absorption spectrum of eosin ($\lambda_{\text{MAX}} = 525$ nm). The emission spectra of the individual oligonucleotide strands labeled with fluorescein and eosin are shown in Figure 8. Annealing of the fluorescein labeled strand to the eosin labeled strand resulted in severe quenching of the donor fluorescence, as shown in Figures 9A and 9B.

As depicted in the above reaction, HIV-IN precisely cleaves the hybrid strand at the junction between CA and GG in the presence of Mg^{2+} , thereby producing a 28-mer donor strand and a 21-mer target strand. Cleavage of the fluorogenic substrate will result in the physical separation of the two fluorophores and subsequent recovery of the quenched donor fluorescence. It was reasoned that the distance dependent process of FRET could be utilized to monitor the DNA cleavage reaction by HIV-IN.

Initially, changes in fluorescence intensity due to cleavage of the substrate by DNase I digestion in the presence of both Mg^{2+} and Mn^{2+} were examined. As shown in Figures 9A and 9B, cleavage of the substrate by DNase I resulted in a dramatic increase in the donor fluorescence intensity. Full recovery of the donor fluorescence for both cations was observed as determined by comparison to the intensity of the fluorescein labeled single-strand. The enhancement of the donor fluorescence was further accompanied by a significant spectral shift due to the increase in the donor emission and reduction in the acceptor emission. These changes are characteristic of resonance energy transfer.

It should be noted that the intensity ratio of the donor fluorescence in the absence and presence of its energy acceptor is approximately 10 fold.

Therefore, one advantage of utilizing FRET is that it provides a method for amplifying the detecting signal. Since HIV-IN usually displays incomplete cleavage activity, the amplification of the signal is an important feature of the fluorescence system. More importantly, it provides a tremendous advantage in terms of its sensitivity and specificity, since this signal amplification increases the signal-to-noise ratio of the fluorescence data.

Furthermore, the DNase I reaction performed in the presence of Mg^{2+} and Mn^{2+} showed that Mn^{2+} results in quenching of the fluorescence. When the spectrum in Figure 9B was peak normalized to that in Figure 9A, no spectral changes were observed (shown in the inset). Therefore, the observed quenching in the presence of Mn^{2+} was the result of a static quenching process of both the donor and acceptor fluorescence. Although Mn^{2+} does not cause complications in interpreting the fluorescence data, it does reduce the sensitivity of the fluorescence detection. Therefore, Mg^{2+} is the preferred divalent cation for fluorescence studies.

The emission spectra shown in Figure 11 illustrate the fluorescence study performed with HIV-IN in the presence of 7.5 mM Mg^{2+} . The increase in donor fluorescence intensity represents DNA cleavage catalyzed by HIV-IN. As mentioned earlier, the shape of the emission spectrum, as a result of cleavage (spectrum B), was different than the spectrum of the substrate without the addition of the enzyme (spectrum A). It should be noted that the shape of the difference spectrum is virtually superimposable with the emission spectrum of donor FITC-labeled single-strand (F-D1/T1). There is a small reduction in the 520-600 nm wavelength region, which represents the eosin emission. These changes are indicative of the reversal of resonance energy transfer and play important roles in discriminating the fluorescence of the cleaved product from that of the substrate, and the quenching mechanism by FRET from quenching by other sources.

As previously mentioned, cleavage of the DNA substrate by DNase I was near completion, whereas HIV-integrase results in only partial cleavage of the DNA substrate. Since the same fluorogenic substrate was utilized, the

intensity ratio of the recovered donor fluorescence (i.e., difference spectra) produced by HIV-IN and DNase I was used to determine the extent of DNA cleavage by HIV-IN. The following equation was used to estimate the extent of cleavage by HIV-IN:

$$[DNA]_c = \frac{F_t - F_0}{F_\infty - F_0} \times [DNA]_i$$

5 where $[DNA]_c$ is the concentration of cleaved DNA, F_t is the fluorescence intensity of time, t , F_∞ is the fluorescence intensity obtained in the presence of DNase I, F_0 is the initial fluorescence intensity, and $[DNA]_i$ is the initial concentration of DNA. Accordingly, the estimated efficiency of the cleavage reaction is 35% in the presence of Mg^{2+} (Figure 10).

10 One of the important features of this fluorescence method is its ability to monitor the data rapidly and continuously. Increases in the data density improve the precision in determining reaction rates required for kinetic analysis. Figure 11A illustrates the kinetics of a continuous fluorescently monitored DNA cleavage reaction by HIV-IN, performed at 37°C. The time-
15 dependent cleavage was monitored with excitation and emission wavelengths of 460 nm and 510 nm, respectively. The excitation wavelength at 460 nm was selected to minimize the direct excitation of the eosin group; changes in the intensity were monitored at 510 nm to avoid the contribution of the emission intensity by the eosin group. An increase in fluorescence intensity was not observed by the addition of integrase in the presence of 20 mM
20 EDTA, indicating that the substrate was not cleaved.

When the reaction was initiated by the addition of integrase in the presence of 7.5 mM Mg^{2+} , a time-dependent increase in fluorescence intensity was observed, indicating the time-dependent cleavage of the substrate.
25 Interestingly, when the cleavage reaction was repeated in the presence of 7.5 mM Mn^{2+} , smaller changes in fluorescence intensity were observed. The data in Figure 11A were intensity normalized to compensate for the fluorescence quenching by Mn^{2+} . At the end of a 60 minute reaction, the changes in the

donor fluorescence were approximately 3 times greater with Mg^{2+} than with Mn^{2+} . As reaction time increased, the differences in enhanced fluorescence intensity became greater. These data indicate that the efficiency of the cleavage reaction was better in the presence of Mg^{2+} than Mn^{2+} , in agreement with our previous data observed by radioactive assays (Lee *et al.*, *J. Biol. Chem.*, submitted 1994).

To verify the fluorescent kinetic data, a time-dependent cleavage reaction with the fluorogenic substrate radiolabeled with ^{32}P at the 5' end of the F-D1/T1 was performed. The reaction products were analyzed by denaturing PAGE followed by autoradiography and quantified by densitometry. As shown in Figure 11B, the gel electrophoresis data and the fluorescence data displayed similar patterns of product formation over the same time course, reconfirming the validity of the fluorescence data. The slightly faster kinetics profile in the radioactive assay was attributed to a small variation in substrate concentration between the two experiments.

Confident interpretation of the fluorescence results reported in this Example relies upon the fluorescence quenching being attributed only to FRET. However, it has been previously reported that fluorescence quenching can be observed by both resonance energy transfer (RET) and a mechanism other than RET. The other fluorescence quenching mechanism was observed when single-stranded DNA containing a single fluorophore was annealed to its unmodified complementary strand (Lee *et al.*, *Anal. Biochem.* 220:377-383 (1994); Clegg *et al.*, *Biochemistry* 31:4846-4856 (1992); Cooper and Hagerman, *Biochemistry* 29:9261-9268 (1990)). This mechanism was discussed in Example 1. This quenching is believed to be due to interactions between the probe and the base of the nucleotide. The fluorescence quenching was also accompanied by a spectral shift. Previous time-resolved studies indicate that the recovered lifetimes were 4.2, 2.7, and 0.5 ns. The spectral shift was associated with the quenching of the 4.2 ns decay component (Lee *et al.*, *Anal. Biochem.* 220:377-383 (1994)). In these previous studies, the

-35-

fluorophore was introduced to DNA via Aminolink 2, which uses a six carbon linker arm.

5 In the present Example, however, the donor fluorophore was introduced to the DNA via the nucleotide analog, 5-amino(12)-2'-deoxyuridine β -cyanoethyl phosphoramidite, which uses a twelve carbon linker arm instead of a six carbon linker arm. Changes in fluorescence intensity were not detected when the fluorescein-labeled D1/T1 strand was annealed to its unmodified complementary strand, D3, nor when the resulting substrate was incubated with DNase I. Time-resolved decay measurements indicated that
10 the recovered lifetimes were a 4.0 ns decay component and a small fraction of a 0.7 ns component. The lifetimes of both the single (F-D1/T1) and double stranded (substrate 2) fluorogenic substrates were the same. This confirms the absence of fluorescence quenching due to probe-DNA interactions. Therefore, the donor fluorescence quenching observed in this Example can be attributed
15 to FRET. This finding, together with Mg^{2+} -dependent endonuclease activity of HIV-IN, made it possible to utilize FRET in a rapid and continuous enzymatic assay system, which will facilitate large-scale screening of integrase inhibitors.

20 The advantages of this fluorescence assay over other assays include its speed, continuity of reaction monitoring, sensitivity, specificity, and capacity for automation through a 96-well fluorescence microplate reader. Variants of this assay are feasible in both cleavage and synthetic reactions, allowing a wider range of future development for FRET based assays in other enzyme systems.

Example 3

Fluorometric Assay for Detecting Nucleic Acid Cleavage Occurring During Catalytic Hybridization Amplification (CHA)

"Catalytic hybridization amplification" (CHA), alternatively known as "cycling probe technology," is described in published PCT application WO 89/09284, and U.S. Patents 5,011,769 and 4,876,187. Briefly, CHA is an improved hybridization assay method whereby the target sequence to be detected is able to capture *many* molecules of the probe in a repeating series of reactions (i.e., "cycling probe"). Essentially, enzyme mediated cleavage of the probe within the probe target duplex results in release of the intact target sequence, which can repeatedly recycle through the reaction pathway. The target sequence serves as a catalytic cofactor for the cleavage of a complementary, labeled nucleic acid probe that is hybridized to the target. The detectable signal in this reaction results from cleavage of the probe, e.g., after repeated CHA cycles, one measures the labeled probe cleavage product. The CHA method is useful in detecting specific DNA or RNA sequences.

The present inventors have reasoned that the last step of CHA (i.e., measuring the labeled probe cleavage product), could be more expeditiously and efficiently carried out by employing the presently disclosed fluorometric assay, based on FRET, for detecting DNA cleavage.

It is expected that the high efficiency of FRET will provide a means to amplify the detection signal. For example, if the donor fluorescence is quenched to 10% of its initial intensity, then complete cleavage of the oligonucleotide substrate (probe) by the RNase H enzyme used in CHA, will result in a 10 fold amplification of the signal. Moreover, only 10% cleavage of the probe will still result in a two fold increase in the detection signal. This intrinsic signal amplification will provide an excellent tool to improve signal-to-noise ratio and thereby increase the confidence in data interpretation.

The cycling probes used consist of DNA-RNA-DNA strands. The first

-37-

fluorescent probe contains a fluorescein labeled nucleotide positioned at one end of the DNA strand and an NH₂-modified nucleotide positioned on the opposite DNA strand. The second fluorescent probe acts as an energy acceptor, and is labeled with at least two fluorophores (eosin and tetramethylrhodamine). The positions of the modified nucleotides will be systematically varied.

The efficiency of FRET of these probes are determined using DNase I and RNase H. Preferably, the fluorphores are placed in close proximity of one another, however, modifications closer to the RNA region may have an effect on RNase cleavage of the probe.

By utilizing the target and the selected probe, and varying the concentrations of the target and the probe, the actual sensitivity of the fluorescence signal will be assessed.

All publications and patents mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patents are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

Having now fully described this invention, it will be understood by those of skill in the art that the same can be performed within a wide and equivalent range of conditions, formulations, and other parameters without affecting the spirit or scope of the invention or of any embodiment therein.

-38-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Georgetown University
- (ii) TITLE OF INVENTION: Fluorometric Assay For Detecting Nucleic Acid Cleavage
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Sterne, Kessler, Goldstein & Fox P.L.L.C.
 - (B) STREET: 1100 New York Avenue, Suite 600
 - (C) CITY: Washington
 - (D) STATE: D.C.
 - (E) COUNTRY: U.S.
 - (F) ZIP: 20005
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: to be assigned
 - (B) FILING DATE: 27-DEC-1995
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/365,473
 - (B) FILING DATE: 30-DEC-1994
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Samuel L. Fox
 - (B) REGISTRATION NUMBER: 30,353
 - (C) REFERENCE/DOCKET NUMBER: 0654.063PC00
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (202) 371-2600
 - (B) TELEFAX: (202) 371-2540

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCCGGATCC ACCC

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid

-39-

- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGGCCTAGG TGGG

14

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 49 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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49

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACTCATGGGC ACACCTTTTA GAGATCGTCA N

31

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 49 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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49

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
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 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE:

-40-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGNCTATGG CGTCCCCTCT G

21

What Is Claimed Is:

1. A method of detecting an enzyme-mediated nucleic acid cleavage reaction in a fluorometric assay comprising the steps of:

5 (a) preparing a fluorescently labeled oligonucleotide containing a nucleic acid sequence recognizable by said enzyme, wherein said oligonucleotide acts as an enzyme substrate;

(b) contacting said oligonucleotide of step a) with said enzyme in an amount sufficient to enzymatically cleave said oligonucleotide; and

10 (c) detecting a nucleic acid cleavage reaction by detecting an increase in fluorescence.

2. The method of claim 1, wherein said oligonucleotide is fluorescently labeled at one end.

15 3. The method of claim 1, wherein said oligonucleotide is fluorescently labeled at both ends.

4. The method of claim 1, wherein said nucleic acid is DNA.

5. The method of claim 1, wherein said nucleic acid cleavage reaction is catalyzed by a restriction enzyme.

20 6. The method of claim 1, wherein said nucleic acid cleavage reaction is catalyzed by a DNase or RNase enzyme.

7. The method of claim 1, wherein said nucleic acid cleavage reaction is catalyzed by a retroviral integrase enzyme.

-42-

8. The method of claim 2, wherein said nucleic acid cleavage reaction is catalyzed by a restriction enzyme.

9. The method of claim 3, wherein said nucleic acid cleavage reaction is catalyzed by a retroviral integrase enzyme.

5 10. The method of claim 5, wherein said restriction enzyme is *BamHI*.

11. The method of claim 7, wherein said retroviral integrase enzyme is HIV integrase.

10 12. The method of claim 1, wherein said enzyme mediated nucleic acid cleavage reaction occurs during a process for amplifying or detecting a specific DNA or RNA sequence.

13. The method of claim 12, wherein said process for amplifying or detecting a DNA or RNA sequence is catalytic hybridization amplification.

15 14. The method of claim 12, wherein said process for amplifying or detecting a DNA or RNA sequence is a polymerase or ligase chain reaction.

1/13

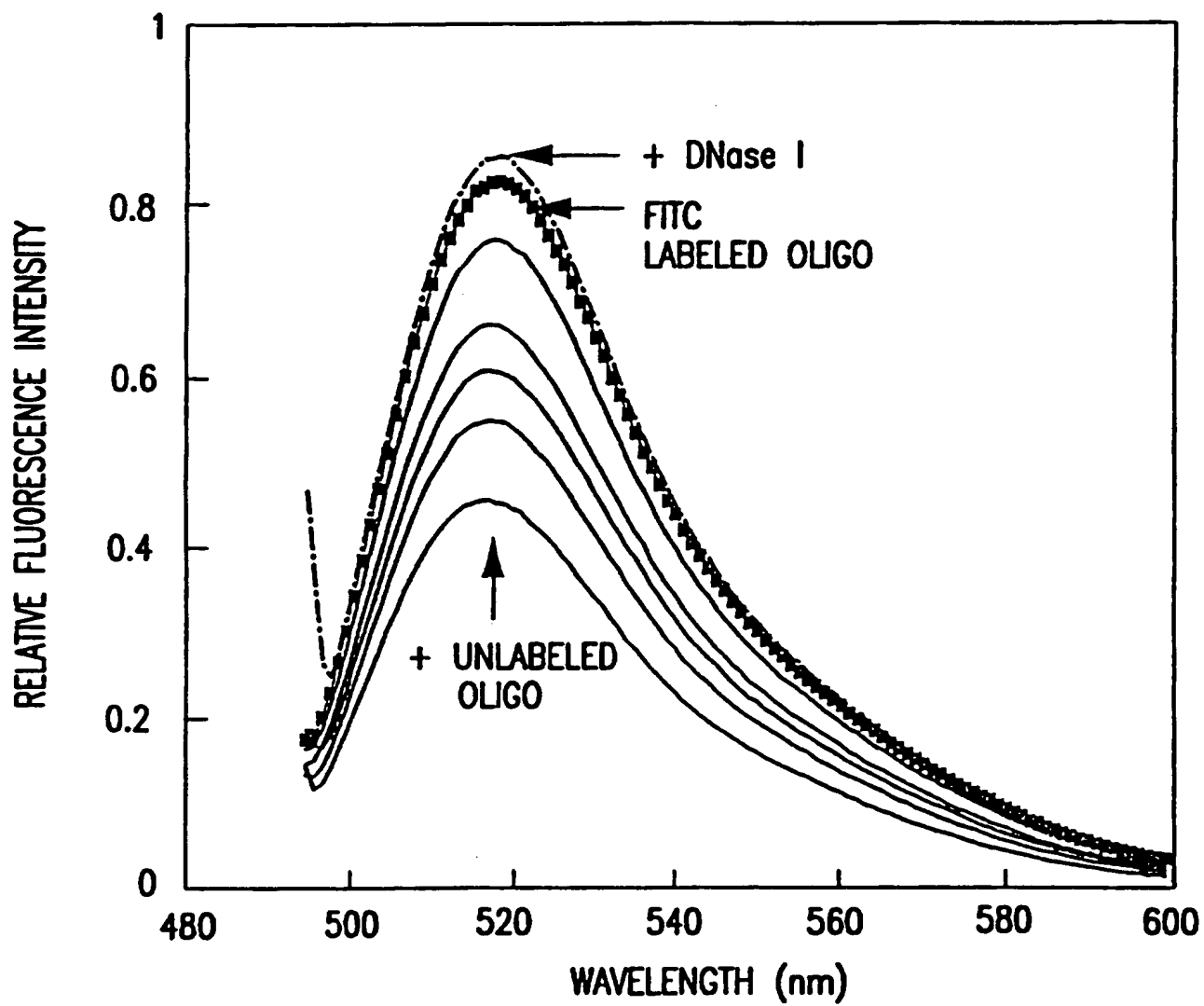


FIG.1

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2/13

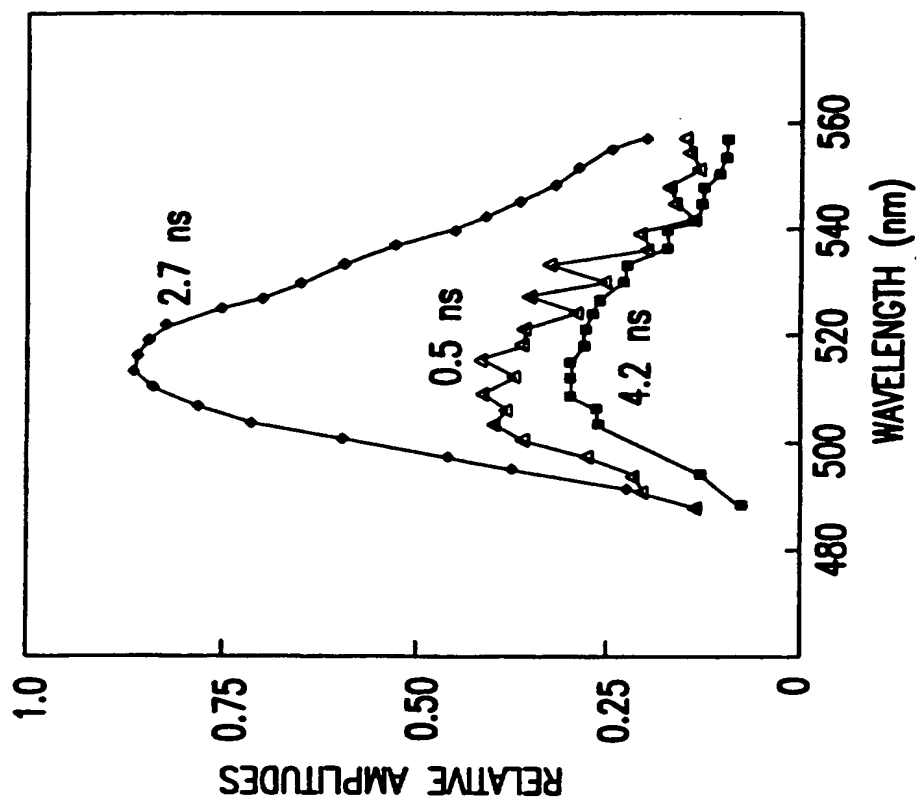


FIG. 2B

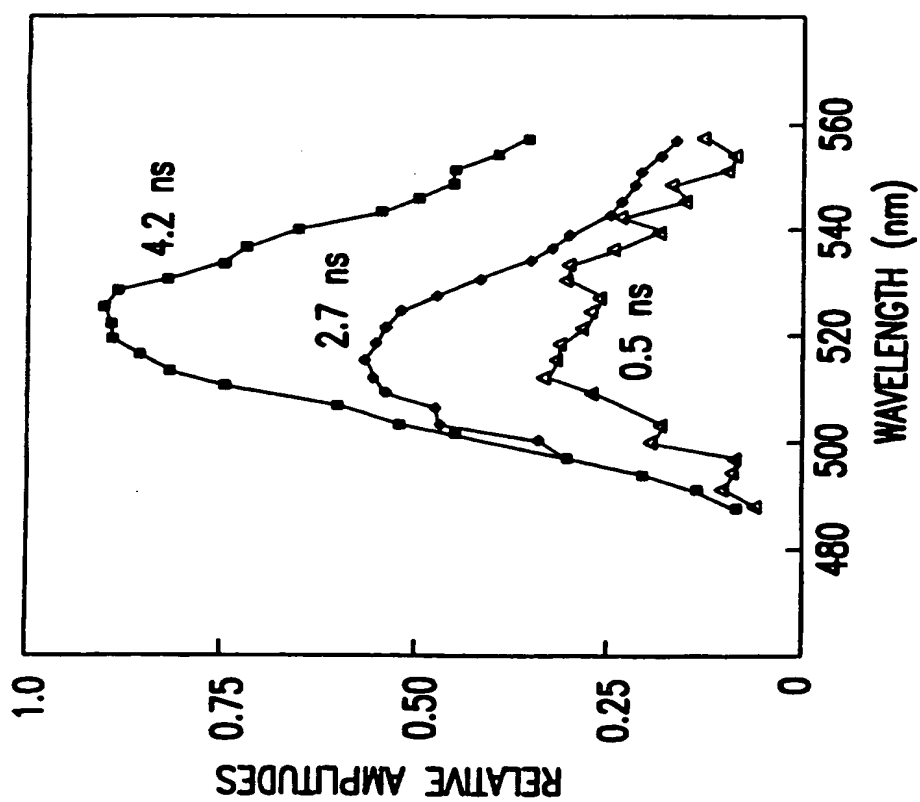


FIG. 2A

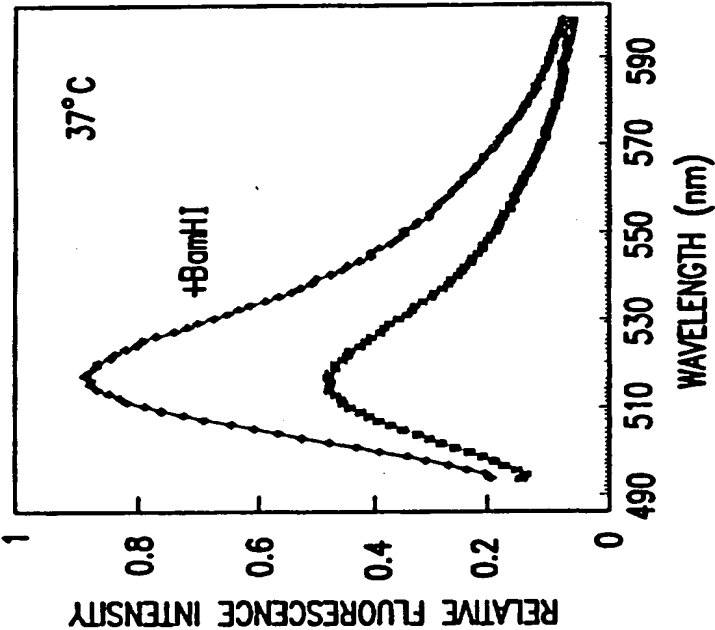


FIG.3B

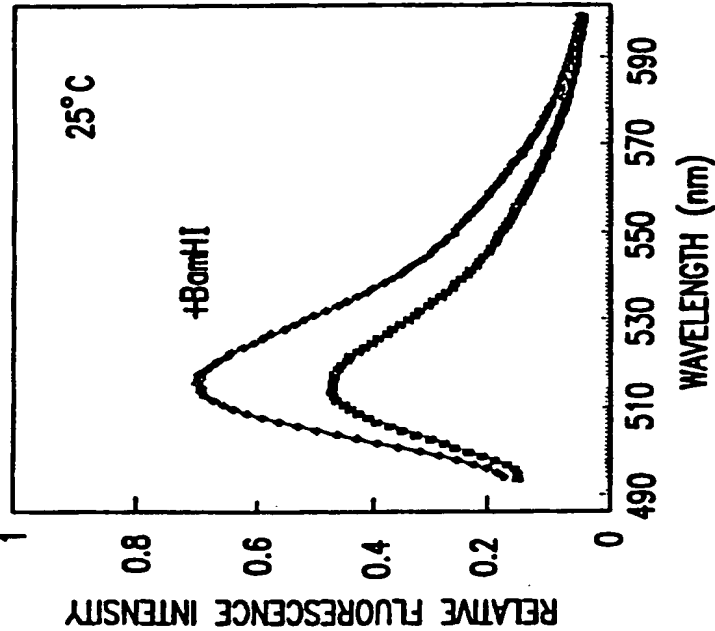


FIG.3A

4/13

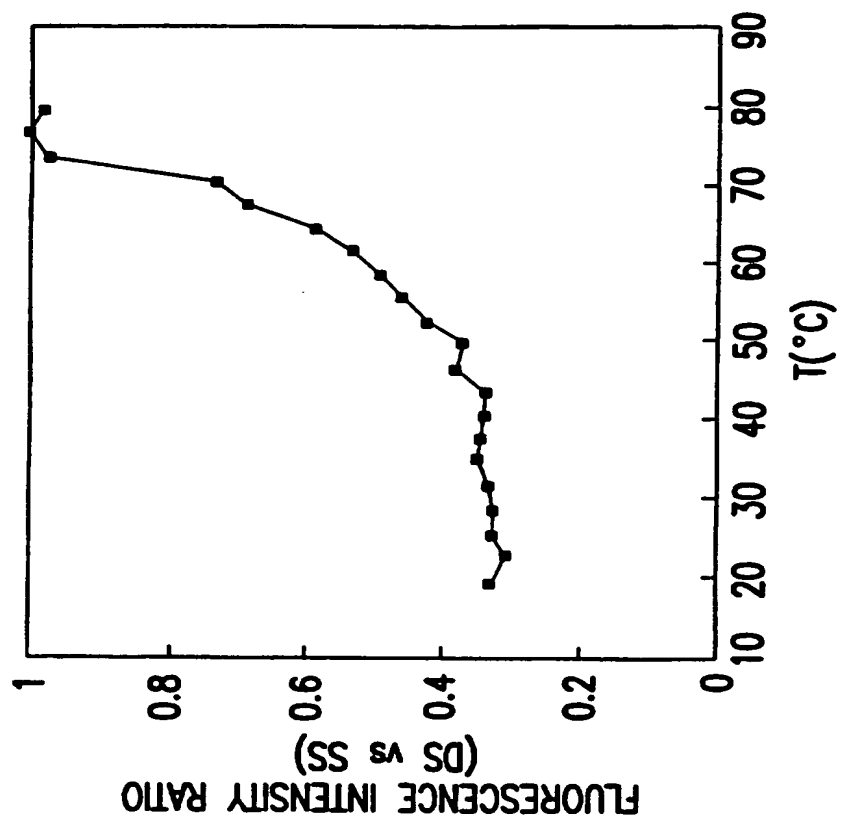


FIG. 5

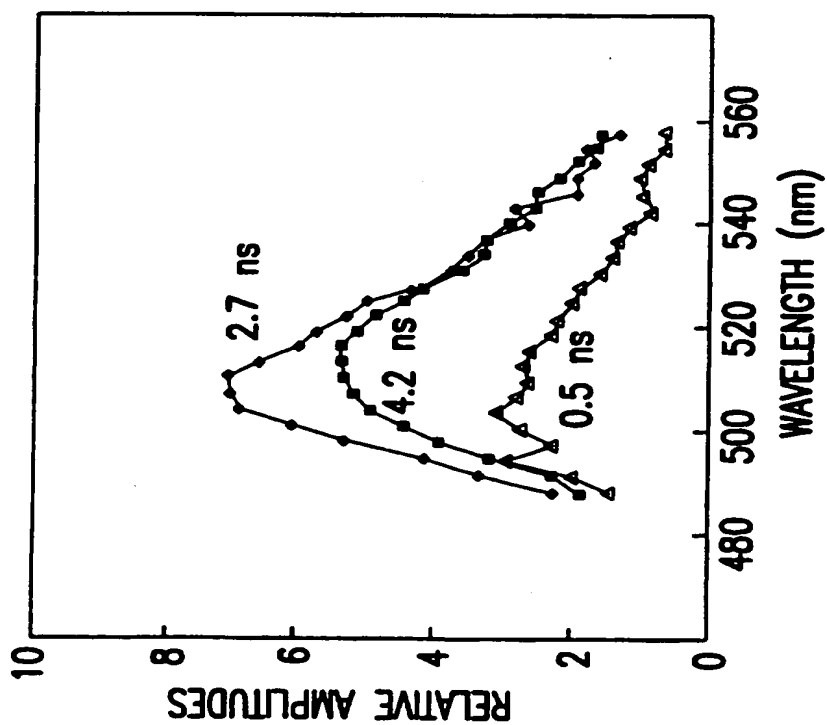


FIG. 4

5/13

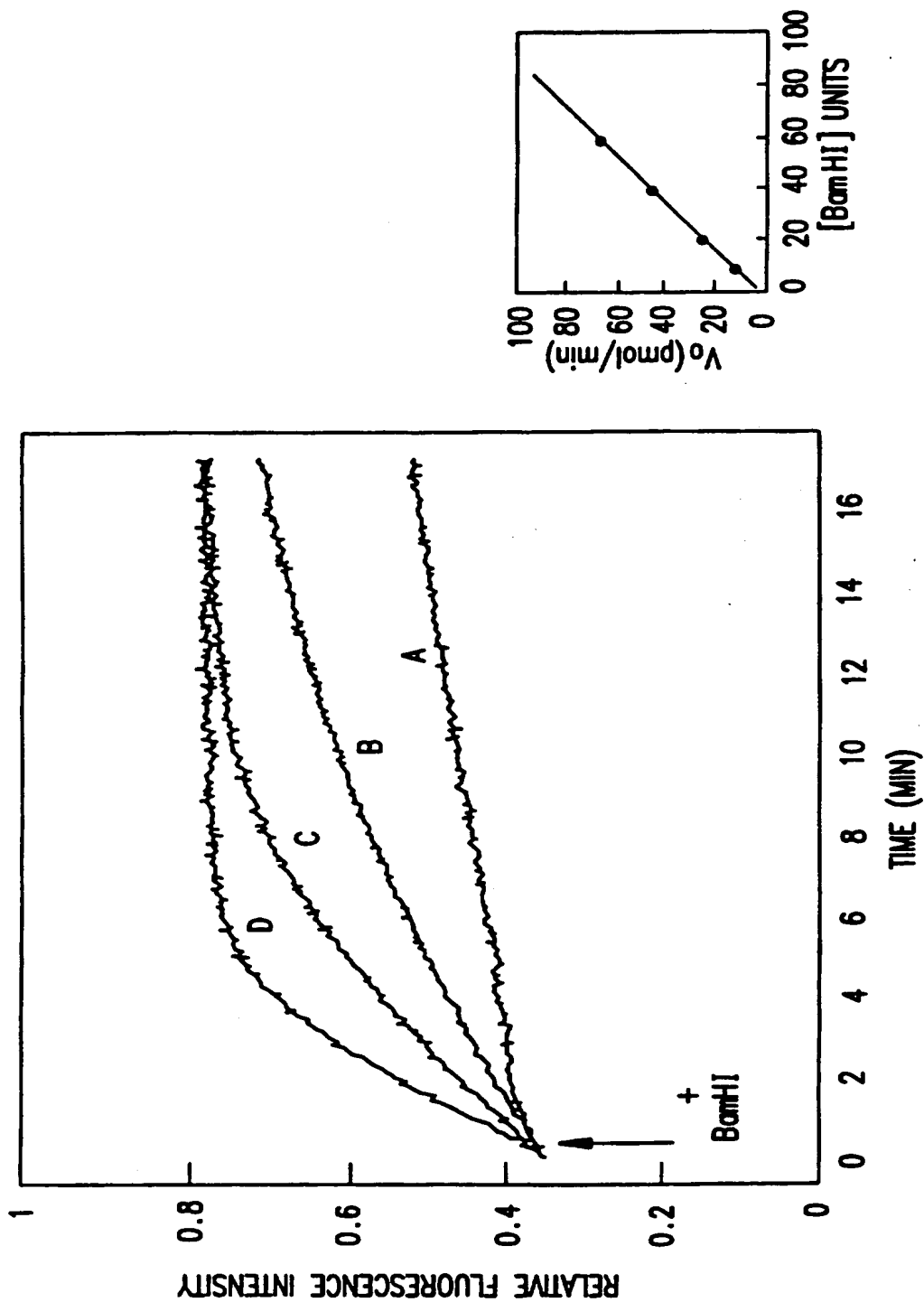


FIG.6B

FIG.6A

6/13

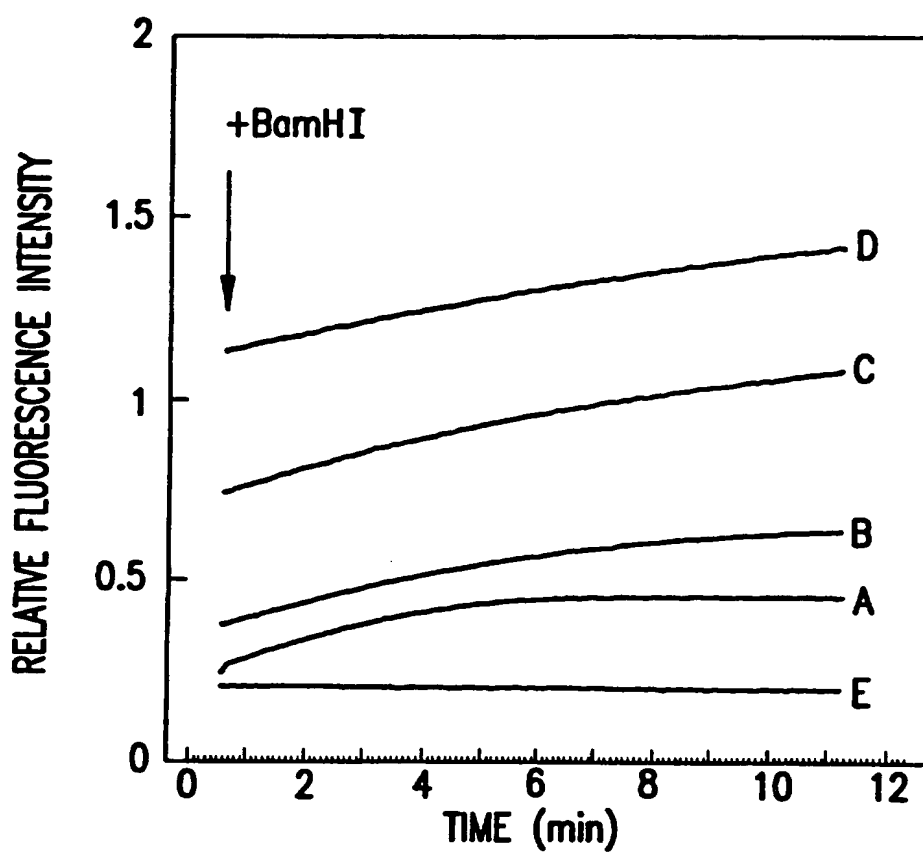


FIG.7A

7/13

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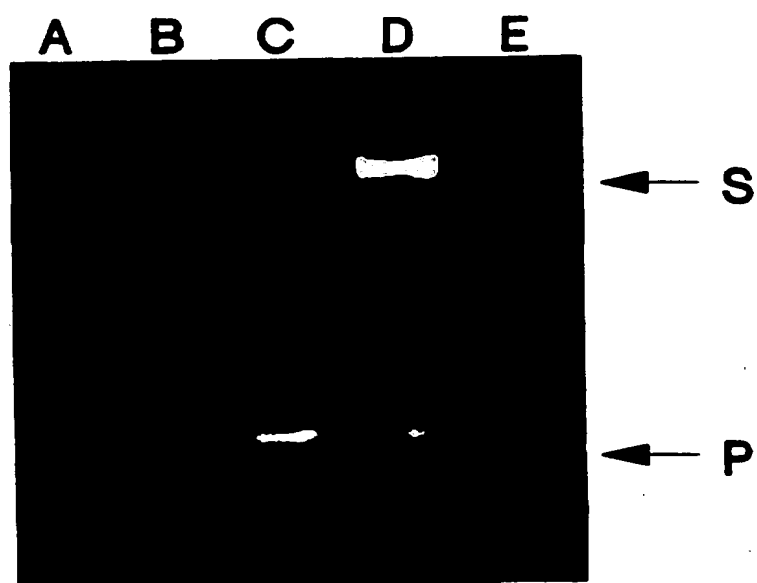


FIG.7B

8/13

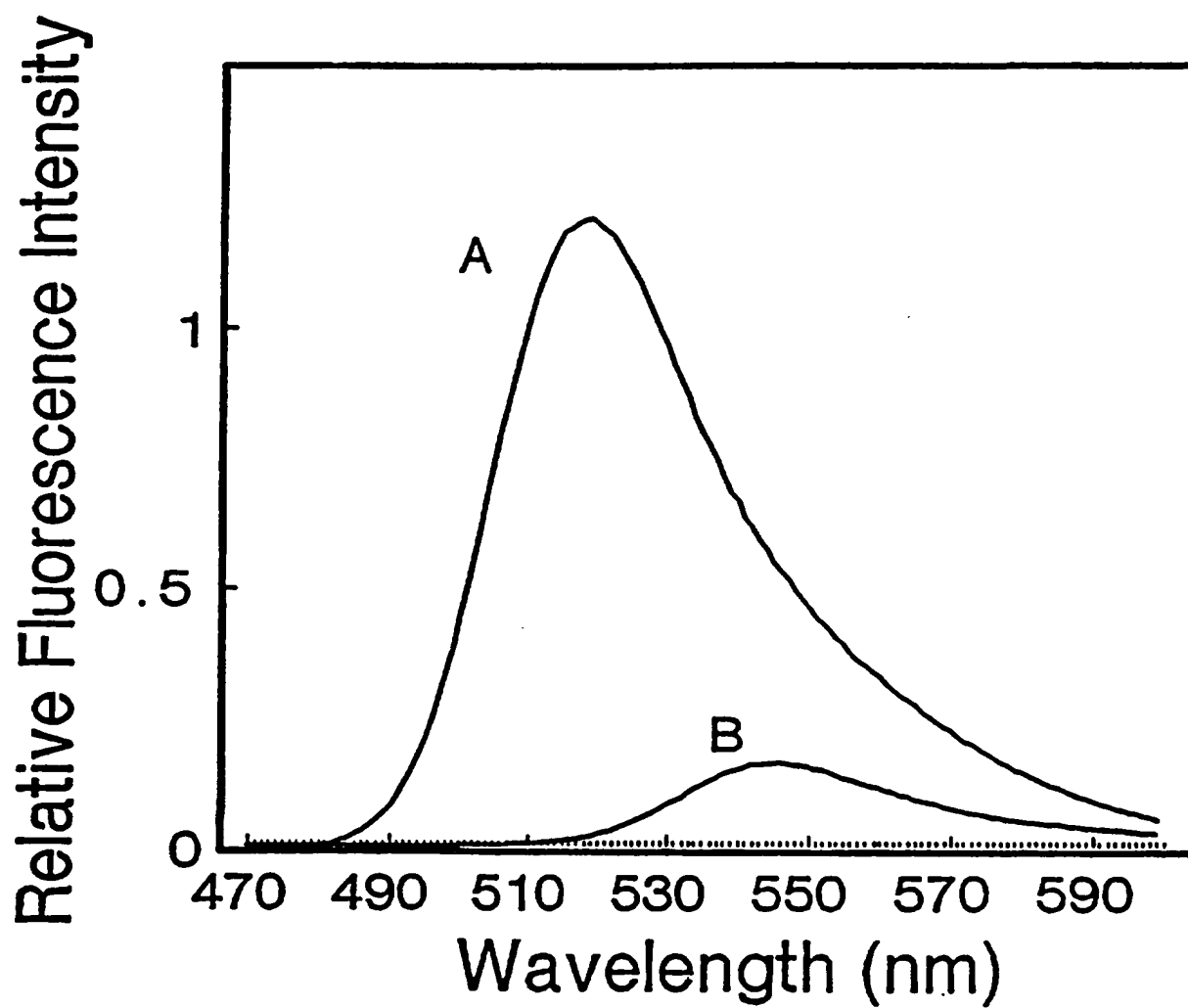


FIG. 8

9/13

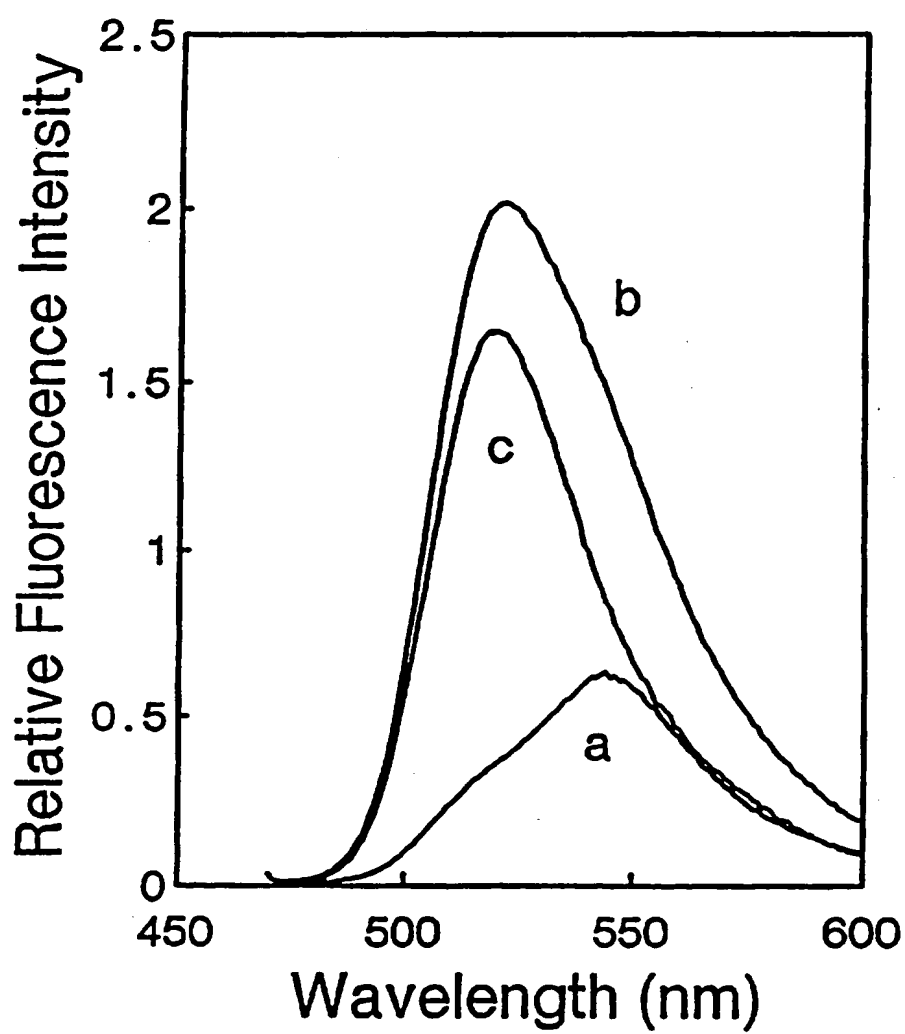


FIG.9A

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10/13

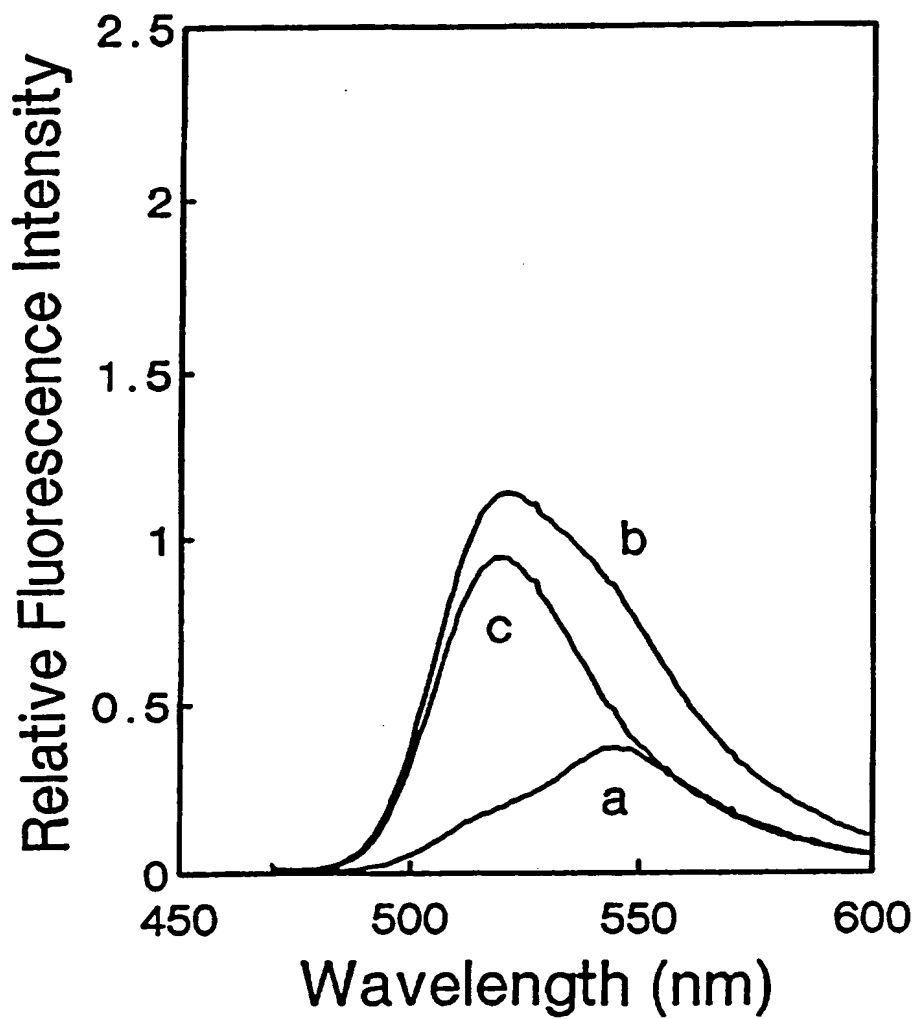


FIG. 9B

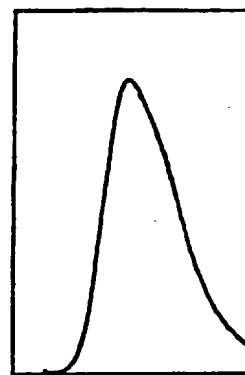


FIG. 9C

11/13

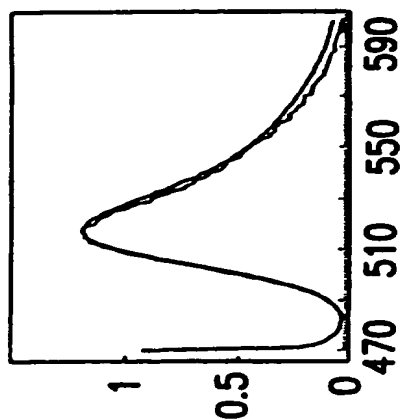


FIG. 10B

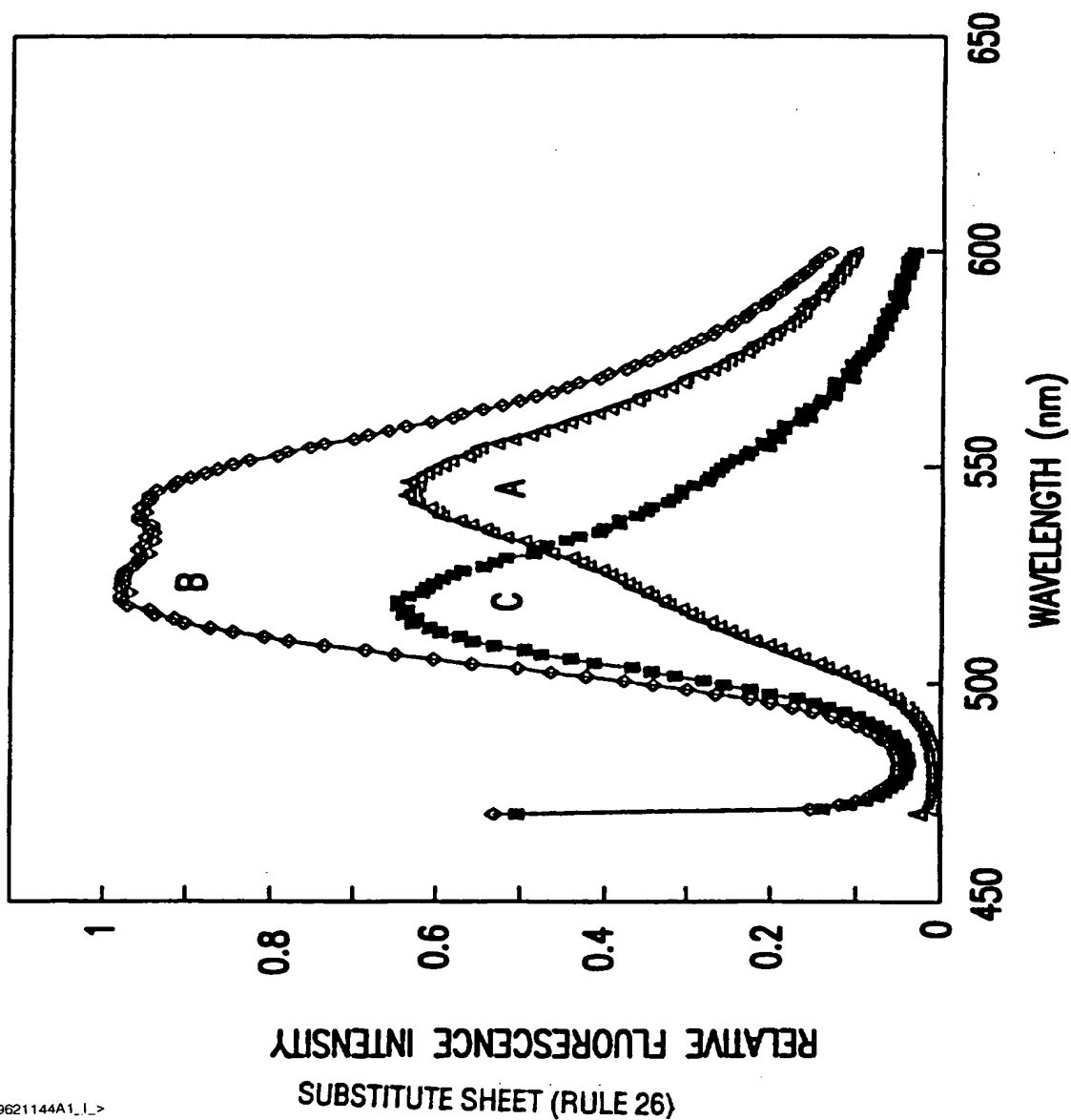


FIG. 10A

12/13

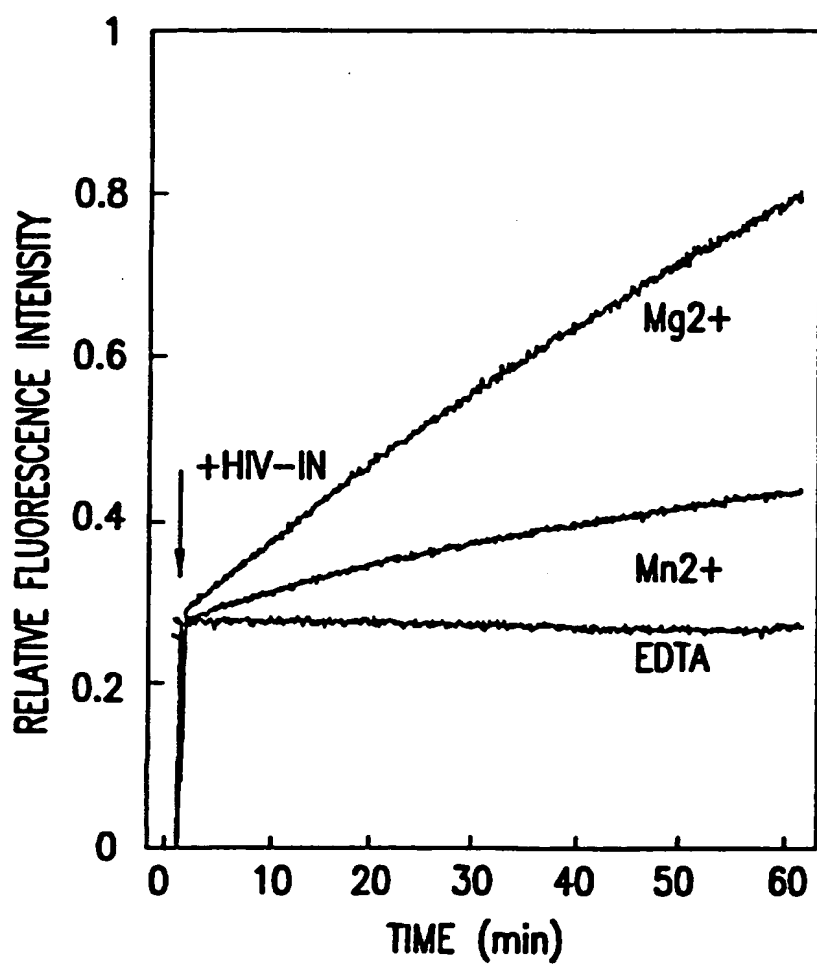


FIG. 11A

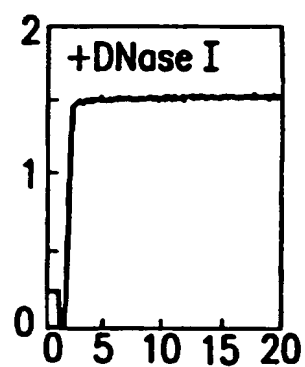


FIG. 11C

13/13

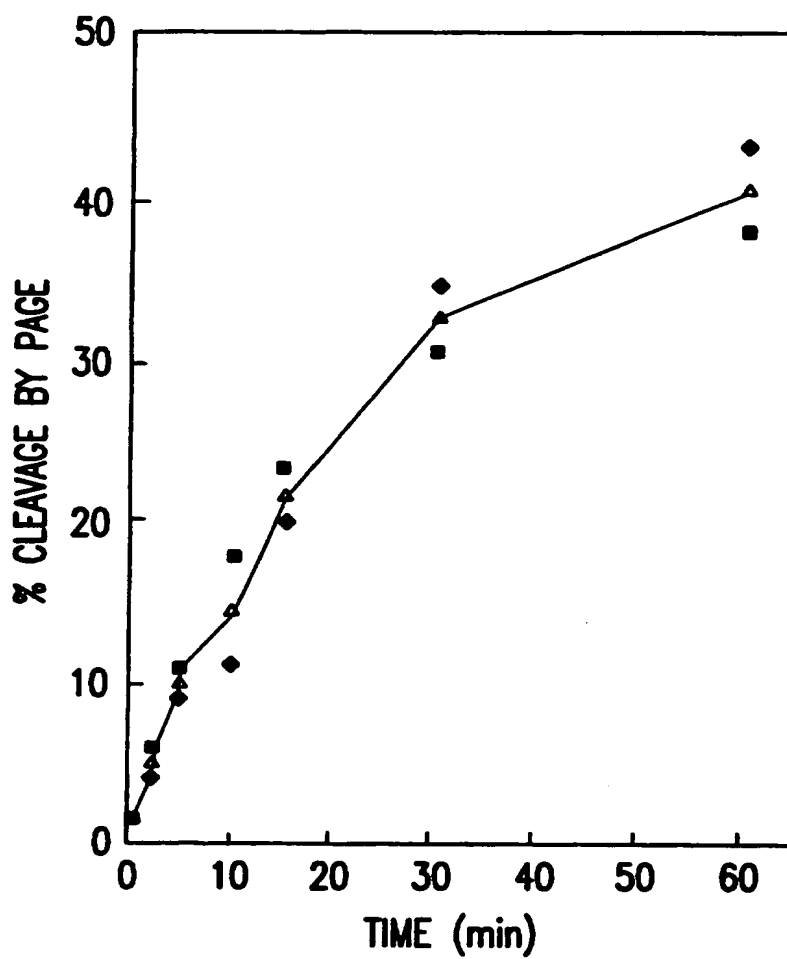


FIG.11B

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16904

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 21/64, 21/76, 33/53, 33/533; C12Q 1/68; C12P 19/34
US CL : 422/82.07; 435/6, 91.2, 968; 436/172, 546

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/82.07; 435/6, 91.2, 968; 436/172, 546

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	Analytical Biochemistry, Volume 220, issued 1994, S.P. Lee et al, "A fluorometric assay for DNA cleavage reactions characterized with <i>Bam</i> HI restriction endonuclease", pages 377-383, especially pages 378-380.	1-6, 8, 10, 12 ----- 7, 9, 11, 13, 14
X ---- Y	Nucleic Acids Research, Volume 22, Number 15, issued 1994, S.S. Ghosh et al, "Real time kinetics of restriction endonuclease cleavage monitored by fluorescence resonance energy transfer", pages 3155-3159, especially 3156 and 3158.	1-5, 8, 12 ---- 6, 7, 9-11, 13, 14
X ----- Y	Analytical Biochemistry, Volume 198, issued 1991, M. Takahashi et al, "Use of a fluorescent DNA analog for fluorometric detection of DNase activity", pages 246-249, especially pages 246-247.	1-4, 6, 12 ----- 5, 7-11, 13, 14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 MARCH 1996

Date of mailing of the international search report

26 MAR 1996

Name and mailing address of the ISA/US
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